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Developmental and Cross-Generational Distribution and Toxicity of Polystyrene Nanoparticles in Zebrafish (*Danio rerio*)

By: Jordan Pitt

Candidate for Bachelor of Science

Department of Chemistry

Work done in Dr. Di Giulio's lab at Duke University

May 2018

APPROVED

Thesis Project Advisor: _____

(typed name of honors advisor below line)

Second Reader: _____

(typed name of second reader below line)

Honors Director: _____

William M. Shields, Ph.D.

Date: _____

Executive Summary

Background

Plastics are ubiquitous in aquatic environments, accounting for 50-80% of marine debris (Barnes et al., 2009). The hazard of macroplastics (particles with a diameter > 5 mm) is well established, with records of countless different species ingesting or becoming entangled in plastic debris (Laist, 1997). Recently, the degradation of macroplastics debris in the ocean and the beach litter into microplastics and nanoplastics has become a significant concern and an increasingly important area of research (Andrady, 2011). Despite this increase in concern relatively little is known about the presence of micro and nanoplastics in freshwater systems or about their potential toxicity to the aquatic organisms dwelling within those systems. This knowledge gap needs to be filled in order to determine the best course of action in limiting any negative effects within aquatic ecosystems.

Nanoplastics are the least well-studied form of plastic debris, with nothing to very little known about their environmental concentrations. Currently there aren't any analytical methods that allow for quantification of nanoplastics in aquatic environments due to their size (Koelmans, 2015). However, nanoplastics are hypothesized to be abundant in aquatic environments due to the deficit of plastic at the lower end of the expected particle size distribution of catalogued marine plastic particles (Cózar et al., 2014).

The presence of these micro- and nano-scale plastic particles in freshwater systems is especially concerning as we don't know the fate, potential bioaccumulation patterns or toxicity of these particles. The ubiquitous nature of plastic debris in aquatic environments makes this a pressing matter that needs further investigation to determine management actions for plastic pollution in addition to the potential long-term ramifications in the exposed aquatic communities.

Purpose

The current study had a dual purpose. First the study examines the uptake, developmental toxicity, and tissue distribution of polystyrene nanoparticles (PS NPs) in zebrafish (*Danio rerio*). The second part of the study examines the effects of a dietary exposure of adult fish on offspring, and whether or not oxidative stress and altered bioenergetics could play a role in polystyrene nanoparticles (PS NPs) toxicity using zebrafish (*Danio rerio*) as a model.

Methods

The first study examined the developmental distribution and toxicity by exposing zebrafish embryos to PS NPs (0.1, 1, or 10 ppm) from 6 hours post-fertilization (hpf) until 120 hpf with observations continuing until 144 hpf. Mortality, hatch rate, deformities, heart rate, mitochondrial function, locomotor activity, and distribution of the PS NPs were the endpoints studied. The second study examined the potential for PS NPs to translocate across different generations. Specifically, adult female and male zebrafish (F0 generation) were exposed to PS NPs via diet for a week. The fish were then bred to produce the F1 generation. Four F1 groups were generated: control (unexposed females and males), maternal (exposed females), paternal (exposed males), and co-parental (exposed males and females). Mortality, hatch rate, deformities, heart rate, mitochondrial function, antioxidant enzyme activity, locomotor activity, and distribution of the PS NPs were the endpoints studied.

Results

The first study examined the distribution and effects of an aqueous exposure of PS NPs on developing zebrafish. PS NPs were found to penetrate the chorion, initially accumulate in the

yolk sac, and migrate to the gall bladder, liver, gastrointestinal tract, head, pericardium, and pancreas of the larvae throughout larval development. This study found that exposure to PS NPs did not induce any significant mortality, deformities, or changes to mitochondrial bioenergetics. Further, the PS NP exposed larvae exhibited bradycardia and hypoactive locomotor activity, a toxicity pattern predicted by the particle localization in the heart and brain. The capability of the PS NPs to affect the physiology and behavior indicates that they may reduce organismal fitness in affected aquatic ecosystems.

The second study examined the distribution and effects of a parental exposure of PS NPs in developing zebrafish. Parental PS NPs dietary exposure did not significantly affect reproductive success. Assessment of tissues from F0 fish revealed that dietary exposure to PS NPs significantly reduced glutathione reductase activity, but did not affect mitochondrial bioenergetics parameters. Assessment of F1 embryos and larvae revealed that PS NPs were present in the yolk sac, gastrointestinal tract, liver, and pancreas of the maternally and co-parentally exposed F1 embryos/larvae. Bradycardia was also observed in embryos from maternal and co-parental exposure groups. In addition, the activity of glutathione reductase and the levels of thiols were reduced in F1 embryos/larvae. Mitochondrial bioenergetics and locomotor activity were not affected in F1 larvae. This study suggests that PS NPs can be transferred via the female gametes from mothers to offspring and that oxidative stress may play a role in PS NPs toxicity.

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Uptake, tissue distribution, and toxicity of polystyrene nanoparticles in developing zebrafish (*Danio rerio*)

Jordan A. Pitt^{1,2,*}, Jordan S. Kozal¹, Nishad Jayasundara^{1,3}, Andrey Massarsky¹, Rafael Trevisan¹, Nick Geitner⁴, Mark Wiesner⁴, Edward D. Levin⁵ & Richard T. Di Giulio¹

¹Nicholas School of the Environment, Duke University, Durham, NC 27708, USA.

²College of Environmental Science and Forestry, State University of New York, Syracuse, NY 13210, USA.

³School of Marine Sciences, University of Maine, Orono, ME, 04469, USA

⁴Department of Civil and Environmental Engineering and the Center for the Environmental Implications of Nano Technology, Durham, NC 27708, USA

⁵Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, NC 27710, USA

***Corresponding author:** jopitt@syr.edu

Abstract

Plastic pollution is a critical environmental concern and comprises the majority of anthropogenic debris in the ocean, including macro, micro, and likely nano-scale (less than 100 nm in at least one dimension) plastic particles. While the toxicity of macroplastics and microplastics is relatively well studied, the toxicity of nanoplastics is largely uncharacterized. Here, fluorescent polystyrene nanoparticles (PS NPs) were used to investigate the potential toxicity of nanoplastics in developing zebrafish (*Danio rerio*), as well as characterize the uptake and distribution of the particles within embryos and larvae. Zebrafish embryos at 6 h post-fertilization (hpf) were exposed to PS NPs (0.1, 1, or 10 ppm) until 120 hpf. Our results demonstrate that PS NPs accumulated in the yolk sac as early as 24 hpf and migrated to the gastrointestinal tract, gallbladder, liver, pancreas, heart, and brain throughout development (48 hpf-120 hpf). Accumulation of PS NPs decreased during the depuration phase (120 hpf-168 hpf) in all organs, but at a slower rate in the pancreas and gastrointestinal tract. Notably, exposure to PS NPs did not induce significant mortality, deformities, or changes to mitochondrial bioenergetics, but did decrease the heart rate. Lastly, exposure to PS NPs altered larval behavior as evidenced by swimming hypoactivity in exposed larvae. Taken together, these data suggest that at least some nanoplastics can penetrate the chorion of developing zebrafish, accumulate in the tissues, and affect physiology and behavior, potentially affecting organismal fitness in contaminated aquatic ecosystems.

Keywords: Behavioral changes; Cardiotoxicity; Plastic pollution; Polystyrene nanoparticles; Uptake and accumulation; Zebrafish (*Danio rerio*).

1. Introduction

Plastics are ubiquitous in aquatic environments, accounting for 50-80% of marine debris (Barnes et al., 2009). While the hazard of macroplastics is well studied, the degradation of macroplastic debris in the ocean and the beach litter into microplastics and nanoplastics has recently become a significant concern and an increasingly important area of research (Andrady, 2011). In fact, while macroplastics are the most widely studied, microplastics and nanoplastics may actually be more pervasive by number in marine environments (Cózar et al., 2014). They also can have a great effect through the food web chain: microplastics have been found to accumulate in zooplankton (Cole et al., 2013), and nanoplastics have been shown to pass from algae to zooplankton and fish (Mattsson et al., 2015a).

Nanoplastics are the least well-studied form of plastic debris, with little known about their environmental concentrations, bioaccumulation potential, or toxicity. Nanoplastics are defined as plastic particles having at least one dimension in the nanoscale (1-100 nm) (Andrady, 2011; Mattsson et al., 2015b). Nanoplastics are hypothesized to be abundant in aquatic environments due to the deficit of plastic at the lower end of the expected particle size distribution (Cózar et al., 2014). The abundance of nanoplastics is likely due to physical and photo-degradation of macro and microplastics in the environment (Andrady, 2011). However, nanoplastics have yet to be quantified in natural systems, because the necessary analytical methods are still under development (Koelmans, 2015).

The biological effects of nanoplastics remain understudied (Rios and Moore, 2007). Most toxicological studies on nanomaterials have focused on metal and carbon based nanoparticles, while very little is known about the toxicity of nanoplastics, such as polystyrene nanoparticles (PS NPs). Polystyrene (PS) is one of the most abundant forms of plastic debris found in the marine environment. For example, PS was the second most common form of floating plastic debris by number found at Tamar Estuary in Southwest England, constituting 25% of the debris (polyethylene was the most common form at 40%) (Sadri and Thompson, 2014). In adult fish, exposure to PS NPs has been shown to compromise immune responses in fathead minnows (*Pimephales promelas*) (Greven et al., 2016) and induce liver lesions in

zebrafish (*Danio rerio*) (Lu et al., 2016). Carboxylated PS NPs have been shown to accumulate in the gut of various marine organisms, such as sea urchin embryos (*Paracentrotus lividus*) and brine shrimp (*Artemia fanciscana*), and as a result it is hypothesized that PS NPs can bio-magnify via trophic transfer (Bergami et al., 2016; Della Torre et al., 2014). Further, it has been demonstrated that PS NPs that accumulate in algae (*Scenedesmus* sp.) can be passed up the food chain and ultimately affect behavior, physiology, and metabolism of Crucian Carp (*Carassius carassius*) (Mattsson et al., 2015a). Thus, fish in the environment are likely exposed to nanoplastics directly through ingestion and through the gills, as well as indirectly via trophic transfer within the food chain (Lu et al., 2016; Mattson et al., 2014).

All of the aforementioned studies focused on the toxicity of PS NPs to adult life stages of fish and other aquatic organisms. In contrast, relatively few studies have focused on the effects of PS NPs on developmental life stages of fish. PS NPs have previously been shown to penetrate the chorion of Japanese medaka (*Oryzias latipes*) embryos (Manabe et al., 2011). However, van Pomeroy et al. (2017) reported that PS NPs did not pass through the chorion of developing zebrafish, though oral exposure played a major role on PS NPs uptake. Additionally, Veneman et al. (2017) reported that injection of PS particles (700 nm) into the yolk sac of 2-day old zebrafish resulted not only in particle distribution throughout the bloodstream and accumulation in the heart region, but also in upregulation of the immunological response, characterized by presence of neutrophils and macrophages around PS particles.

To this end, the present study aimed to assess the potential bioaccumulation and toxicity of PS NPs in embryonic and early larval stages of zebrafish. We predicted that the PS NPs would pass through the zebrafish chorion since the particles are smaller than the pore size (0.6-0.7 μm) and were shown to penetrate the chorion of medaka (Kirsten, 2011; Manabe et al., 2011). Further, we hypothesized that PS NPs would accumulate in various regions, including yolk sac, pericardium, gallbladder, and gut, similarly to previous studies (Manabe et al., 2011; Skjolding et al., 2017; Veneman et al., 2017). Finally, given that PS NPs have been shown to accumulate at multiple tissues throughout the development, we predicted that PS NPs could alter zebrafish physiology, bioenergetics, and behavior (Chen et al. 2017, Mattson et al. 2014).

2. Materials and methods

2.1 PS NPs

PS NPs (cat. #FSDG001) were purchased from Bangs Laboratories, Inc. (Fishers, IN, USA). The stock solution contained 1% fluorescent (internally labeled with Dragon Green; ex./em. 480/520) PS NPs with a nominal mean diameter of 51 nm. According to the manufacturer, internal labeling does not change the particle surface chemistry, and thus should not affect their adsorptive and bioaccumulative properties. Additionally, the stock solution contained up to 0.1% sodium dodecyl sulfate (SDS) and 0.09% sodium azide. An aliquot of stock solution was sonicated for 60 s at 50/60 Hz using NEY Ultrasonic Cleaner ULTASONIK 2QT/H (Barkmeyer Division Yucaipa, Ca, USA) prior to preparation of the working solutions (see section 2.4).

2.2. PS NPs characterization

PS NPs were characterized using a dynamic light scattering (DLS) (Zetasizer Nano, Malvern Instruments Ltd., Malvern, UK). The hydrodynamic diameter and zeta potential of 5 ppm PS NPs were assessed in the exposure medium (see section 2.4).

2.3. Zebrafish husbandry and embryo collection

Laboratory reared AB wild-type (EkkWill Waterlife Resources; Ruskin, FL) *D. rerio* were maintained in a recirculating AHAB system (Aquatic Habitats, Inc., Apopka, FL, USA) on a 14:10 h light/dark cycle. Water quality was maintained at 28-29°C, pH 7.0-7.5, and 60 ppm artificial seawater (ASW; Instant Ocean, Foster & Smith, Rhinelander, WI, USA). The fish were fed twice daily with brine shrimp (INVE Aquaculture, Inc., Salt Lake City, UT, USA) in the morning and Zeigler's Adult Zebrafish Complete Diet (Aquatic Habitats, Inc.) in the afternoon. Breeding crosses (3 females: 2 males) were set at 5 PM, and embryos were collected the following morning within 1 h of spawning between 9 and 10 AM and kept in 30% Danieau's medium (17.4 mM NaCl, 0.21 mM KCl, 0.12 mM MgSO₄, 0.18 mM

Ca(NO₃)₂, and 1.5 mM HEPES at pH 7.6) at 28°C. All zebrafish care and husbandry procedures were approved by the Duke University Institutional Animal Care and Use Committee (A139-16-06).

2.4. Experimental set-up

At 6 h post-fertilization (hpf), embryos were randomly assigned to 20 mL glass scintillation vials with 2 embryos per vial. Each vial contained 7.5 mL of 65 ppm ASW supplemented with 0.00003% methylene blue. The 65 ppm ASW medium used is similar to zebrafish egg water established by Westerfield (2000). The embryos were randomly sorted into 4 groups: 0 (control), 0.1, 1, and 10 ppm PS NPs; these concentrations are up to 2 orders of magnitude lower than the concentrations used in a previous zebrafish embryo study (van Pomeroy et al., 2017). The vials also contained 0.0001% SDS and 0.00009% sodium azide for the 10 ppm PS NPs solution with decreasing orders of magnitude for the 1 and 0.1 ppm solutions from the stock PS NPs solution (SDS and sodium azide are included in the stock solution to prevent aggregation and bacterial growth, respectively). Such concentrations of SDS and sodium azide did not significantly affect mortality, hatching success, heart rate, pericardial area, and uptake of PS NPs. Inclusion of methylene blue also did not significantly affect the toxicity of PS NPs (see sections 1 and 2 of Supplemental Materials for details). The embryos/larvae were exposed to PS NPs until 120 hpf without refreshing the exposure medium. Observations continued from 120 hpf until 168 hpf in 65 ppm ASW medium without PS NPs in order to assess the relative amount of PS NPs after the exposure. The toxicity endpoints were repeated using at least 3 independent zebrafish cohorts (see details below).

2.5. General physiology

Survival rate was assessed every 24 h from 24 to 120 hpf, and dead organisms were removed. A total of 240 embryos were used per group. Hatching rate was assessed at 72 hpf by the inspection of a total of 50 embryos per group. Both the survival and hatching rates were calculated from at least 3 independent experiments (n=3-8), which were based on cohorts from separate breeding events. Heart rate

was measured at 72 hpf using tricaine as an anesthetic agent [125 mg/L in 30% Danieau's medium; a concentration shown not to have a notable effect on embryonic heart rate (Huang et al., 2010)]. The larvae were washed and placed into the tricaine solution for 5 min. Following this, heart rate was individually evaluated by counting beats for 15 sec in 5 randomly selected larvae from different vials in each treatment group in 3 cohorts from separate breeding events (n=15). Larval deformities were analyzed using bright field images that were taken during the fluorescence imaging of the larvae (see section 2.6). Deformities were analyzed at 24 h intervals from 48 hpf to 168 hpf. At every time point, 5 individuals from each treatment group were imaged (n=5). Specifically, presence or absence of spinal curvature and craniofacial abnormality were assessed in a blind fashion. Pericardial area was quantified in 5 larvae per treatment group (n=5) at 72 hpf using ImageJ software (National Institutes of Health, Bethesda, MD, USA).

2.6. Uptake and distribution of PS NPs

Zebrafish were imaged using Zeiss Axioskop (Carl Zeiss, Oberkochen, Germany) at 24 h intervals from 24 hpf to 168 hpf. At every time point, 5 randomly selected individuals from each treatment group were imaged (n=5). While the zebrafish were exposed with their chorions intact, the 24 hpf embryos were imaged both pre and post enzymatic dechoriation with pronase (1 mg/mL). Fluorescence images were captured using a 75W/2 xenon short-arc lamp, a Photometric CoolSNAPfx monochrome CCD camera (Roper Scientific, Tuscon, AR, USA), 480/30 nm (emission) and 535/40 nm (ex) filters, and 100 ms camera exposure. Organ placement in the larvae was analyzed using illustrations from Wallace and Pack (2003). Fluorescence intensity was analyzed using ImageJ software. The brightness/contrast of fluorescent images was adjusted to the brightness/contrast of the fluorescent signal corresponding to 10 ppm PS NPs. The fluorescence values were normalized to the control group and expressed as fold change. Due to the high difference in fluorescence levels between the exposure groups (up to 250 fold), log transformed values were used in the statistical analysis.

2.7. Larval behavior

At 120 hpf, zebrafish larvae were washed and transferred into beakers with 200 mL of fresh media. The larvae were transported to the behavioral testing facilities, where they were kept on a 14:10 h light/dark cycle at 28 °C. At 144 hpf, around 12 pm, the larvae were transferred into a 96 well plate (1 larvae/well, 23-24 wells/treatment group, in 3 cohorts from separate breeding events, n=70-72). The larvae were allowed to acclimate for an hour in the dark, prior to being transferred to a DanioVision™ observation chamber (Noldus Inc., Wageningen, The Netherlands) for an alternating light/dark test (Massarsky et al., 2015; Brown et al., 2016; Bailey et al., 2016). This is a 50 minute test where the first 10 minute period is habituation in the dark, and then the subsequent 40 minutes consist of 2 cycles of alternating 10 minute periods of light and dark. Video data were recorded at a sample rate of 30 times/second via a high-speed infrared camera. The EthoVision XT® software (Noldus, Wageningen, The Netherlands) was used to calculate total distance moved for each individual larvae throughout the test.

2.8 Bioenergetics

The oxygen consumption rate (OCR) was assessed using the XFe24 Extracellular Flux Analyzer (Agilent Instruments, CA, USA). This bioenergetic profiling method was adapted from Stackley et al. (2011). For this assessment, embryos (24 hpf) were staged in a 24 well plate with 700 µL of 65 ppm artificial seawater (2 embryos/well, 5 wells/exposure group, 4 blank wells). Carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) and sodium azide were loaded into separate injection ports, resulting in working concentrations of 2.5 µM and 6.25 mM in the well, respectively. The plate was then inserted into the Analyzer where 8 OCR measurements (basal OCR) were first taken before the FCCP was injected into the wells (8 measurements) followed by the sodium azide injections (25 measurements). The FCCP acts as a mitochondrial un-coupler allowing for measurement of maximal respiration, and the sodium azide inhibits cytochrome c oxidase function allowing for measurement of non-mitochondrial respiration. The total basal OCR was calculated by averaging the three lowest values from the basal

measurements. The OCR in the presence of pharmaceutical agents was calculated by averaging the three highest values for the FCCP treatment (total maximal) and by averaging the three lowest values for the sodium azide treatment (non-mitochondrial). These OCR measurements were used to calculate additional respiratory parameters, including basal mitochondrial, maximal mitochondrial, and mitochondrial reserve capacity. The extracellular acidification rate (ECAR) was detected concurrently with the OCR measurements. This parameter is used as a proxy for glycolytic activity. This allowed for the calculation of the total basal ECAR and OCR/ECAR values, which estimates the degree to which the embryo utilizes oxidative phosphorylation versus glycolytic activity.

2.9. Statistical analysis

For fluorescence imaging and deformity endpoints, one-way or two-way Analysis of Variance (ANOVA) with a Fisher's LSD post hoc method were used to assess statistical differences among treatment groups. Such analyses were carried out with log transformed values for fluorescence endpoints. For heart rate, two-way ANOVA with treatment and cohort factors with a Fisher's LSD post hoc method was used to test for PS NPs treatment effects. These analyses were conducted using Graph Pad Prism 6.0 (San Diego, CA, USA). For larval behavior, mixed-design repeated-measures ANOVA with treatment group and cohort as the between-subject factors and light condition and trial minute as the repeated measures was used with a Dunnett's two-tailed post hoc to test for PS NPs treatment effects. This analysis was conducted using StatView 5.0.1 (SAS Institute, Cary, NC). For all endpoints, p-value <0.05 was considered statistically significant.

3. Results

3.1. PS NPs characterization

The PS NPs (5 ppm in ASW medium) ranged from 20 to 100 nm in diameter with a mean hydrodynamic diameter of 34.5 ± 10.8 nm. The zeta potential was -21.1 ± 2.47 mV. Both values indicate a low aggregation behavior for these PS NPs.

3.2. General physiology

Mortality was not significantly different between the exposure groups throughout embryonic and larval development at any of the time points measured (24-120 hpf) (Fig. 1A). The hatching success was also not significantly different between exposure groups (Fig. 1A). Exposure to PS NPs did not induce deformities nor did it increase the pericardial area (Fig. 1B). In contrast, heart rate was significantly decreased in larvae from all exposure groups relative to control (Fig. 1C). Specifically, heart rate decreased in a dose-dependent manner by 5%, 8%, and 10% in the 0.1, 1, and 10 ppm PS NPs treatment groups relative to controls, respectively.

3.3. Uptake and distribution of PS NPs

Green fluorescence was used to qualitatively and quantitatively evaluate uptake and distribution of PS NPs in zebrafish embryos and early larval stages between 24 hpf to 168 hpf. At 24 hpf, fluorescence was mainly observed in 1 and 10 ppm PS NPs exposed embryos in the yolk sac with some fluorescence also observed in the head in the 10 ppm group (Fig. 2). Aggregation of the PS NPs was visible on the surface of the chorions of some exposed embryos (Fig. 2A). At 24 hpf, the fluorescence of the yolk sac region was significantly higher in zebrafish embryos exposed to 1 and 10 ppm PS NPs relative to the control (11 and 102 fold higher, respectively) (Fig. 2C).

At 48 hpf and 72 hpf, the fluorescence levels were significantly higher in the yolk sac and head (presumably the brain) even at the lowest concentration (Fig. S3 & S4). At these same time points, fluorescence was also strongly observed in the pericardium at 1 and 10 ppm PS NPs (Fig. S3 & S4).

By 120 hpf, the larva has resorbed the yolk sac, and the gastrointestinal (GI) tract is functional. At this stage in larval development, fluorescence was higher in the GI tract, pancreas, and the head for all the

exposure groups relative to controls. At 1 ppm and 10 ppm PS NPs, fluorescence was also higher in the liver, gall bladder, and pericardium (Fig. 3). The magnitude of the fluorescence in the GI tract and the pancreas appeared to be relatively constant after removal of the larvae from the exposure medium (144 hpf – Fig. S5, and 168 hpf – Fig. 4); however, the fluorescence in the head, gall bladder, liver, and pericardium began to decrease in all exposure groups during the depuration. Despite the decrease, the fluorescence levels in all the tissues analyzed from larvae exposed to 10 ppm PS NPs remained significantly higher than those of the controls (Fig. S5, Fig. 4). Fluorescence levels remained significantly higher in the GI tract and pancreas in larvae exposed to 1 ppm PS NPs, and they returned to control values in all the analyzed organs in the lowest exposure group (Fig. S5, Fig. 4).

The fluorescence values of each analyzed tissue during the exposure and depuration periods are summarized in Figure 5. PS NPs quickly accumulate in the yolk sac, head, and pericardium by 48 hpf, with the highest fluorescence levels in the pericardium. Between 72 and 120 hpf, fluorescence levels start to decrease in these organs, a possible sign of PS NPs excretion. During the depuration period (144-168 hpf), fluorescence levels continue to decrease in the head and pericardium, as well as gall bladder and liver. As previously mentioned, fluorescence levels remain stable and significantly higher than controls in the GI tract and pancreas only at 1 and 10 ppm PS NPs.

3.4. Larval behavior

Larval locomotor activity over the course of an alternating light/dark test was measured at 144 hpf, following a 24 h acclimation in clean embryo medium. PS NPs exposure significantly reduced larval locomotor activity (Fig. 6). Specifically, there was a significant main effect of PS NPs treatment on total distance traveled over the course of the alternating light-dark test. This effect was driven by significant hypoactivity in larvae from the medium (1 ppm) PS NPs group ($p < 0.05$) relative to control throughout the trial (Dunnett's two-tailed post hoc test). As expected, there was a significant main effect of light condition ($p < 0.0001$) on locomotor activity, with larvae from all treatments swimming more in the dark than the light. There was not a significant interaction of light condition and PS NPs treatment ($p < 0.98$).

However, while significant hypoactivity relative to control was measured in the 1 ppm PS NPs exposed larvae during the initial habituation period in the dark ($p < 0.05$) and the alternating dark periods ($p < 0.02$), this effect was trending but not significant in the alternating light periods ($p < 0.11$) (Fig. 6A & B). While not significant, the larvae exposed to 0.1 or 10 ppm PS NPs also appeared to be hypoactive relative to the control group. Notably, there were significant interactions of PS NPs treatment and cohort during both the acclimation period ($p < 0.02$) and the alternating light-dark test ($p < 0.04$), reducing the interpretability of these data.

3.4. Bioenergetics

The basal, maximal, and non-mitochondrial OCR values were not significantly different across treatment groups. The calculated values for basal mitochondrial, maximal mitochondrial, and mitochondrial reserve were also not different. No significant differences were noted for ECAR or the ratio of basal OCR to ECAR (Fig. S6).

4. Discussion

While the hazards associated with macro and microplastics to aquatic organisms are relatively well characterized, the bioaccumulation and toxicity of nanoplastics are only beginning to be considered even though they could potentially be more hazardous (Koelmans, 2015). The current study provides strong evidence that PS NPs are taken up by developing zebrafish, localize to specific tissues, and potentially exert toxic effects that are suggested by their tissue localization pattern. The following sections discuss our results in detail and consider the potential physiological and ecological implications associated with exposure to PS NPs, which are likely a ubiquitous contaminant in aquatic environments.

First, we demonstrate that PS NPs are able to penetrate the zebrafish chorion, which is a known physical barrier to some chemicals (Mizell and Romig, 1997). The PS NPs initially accumulate in the yolk sac and the head, and later in other regions, including pericardium, gall bladder, pancreas, liver and

GI tract. These results are consistent with the findings of analogous studies in medaka (Manabe et al., 2011; Kashiwada, 2006), but are in contrast with those of van Pomerén et al. (2017), who reported that the chorion was an effective barrier against PS NPs. These discrepancies are likely due to differences in PS NPs concentration (our study used lower concentrations), supplier (and/or synthesis method) of the PS NPs, and the size of the particles [in our study the particles were approximately half the size of those used by van Pomerén et al. (2017)]. The ability of PS NPs to penetrate the chorion suggests that accumulation in aquatic environments could affect development of fish species and potentially have long lasting impacts at the population level.

While nanoparticles have a tendency to aggregate and PS NPs aggregates were occasionally observed on the outside of the chorion, there was high fluorescence visible within the yolk sacs of the dechorionated embryos as early as 24 hpf. This suggests that the chorion may act as a barrier against PS NPs aggregates but does not exclude PS NPs at the studied size (34.5 nm mean diameter). Further, the yolk sac appears to be a main target during the initial PS NPs uptake. The yolk sac is the nutrient reserve for the developing zebrafish, containing lipids that are processed and mobilized by lipoproteins and are essential for metabolic and developmental processes (Fraher et al., 2016). PS NPs have been shown to interact with lipid membranes (Rossi et al., 2014), and PS microparticles have been shown to alter lipid metabolism and induce hepatic lipid accumulation in zebrafish (Lu et al., 2016). Notably, multiple nuclear receptors involved in lipid metabolism are upregulated following microinjection of 700 nm PS particles into the yolk sac of zebrafish (Veneman et al., 2017). Early life exposure to PS seems to affect lipid metabolism in zebrafish, which could indicate that PS NPs have the potential to induce metabolic disorders. It is also possible that the high lipid content of the yolk sac could be a target for PS NPs accumulation, where further lipid mobilization could play a role on the distribution of PS NPs throughout the embryo/larva.

Despite the apparent ability of PS NPs to penetrate the chorion, PS NPs had no effect on mortality, hatching success, deformities, or pericardial area. However, zebrafish exposed to PS NPs exhibited significant bradycardia. Notably zebrafish from all exposure groups (0.1, 1, and 10 ppm) had

significantly reduced heart rates relative to controls, suggesting that changes in heart function occur even at relatively low PS NPs concentrations. A number of factors may play a role in reduced heart rates. One possibility is that PS NPs may interact with cardiac sarcomeres. Previous findings suggest that PS NPs are localized into cells and are not membrane bound (Geiser et al., 2005) creating potential for interactions with cardiac sarcomeres affecting heart rate. Another possibility is that oxidative stress is occurring, as suggested previously (Chen et al. 2017), altering the function of the heart. Future studies will be necessary to determine the mechanism by which PS NPs reduce heart rate. PS NPs-induced bradycardia may have negative implications for fish in the aquatic ecosystems where cardiac function and aerobic condition may influence predator prey interactions and other factors important for organismal ecological fitness.

Further, the present study is the first to show that PS NPs also localize to the GI tract, heart, head, liver, and pancreas as the zebrafish develops and the yolk sac is resorbed. Fluorescence was detected even at the lowest PS NPs concentration (at the ppb range), highlighting the potential bioaccumulation of these compounds throughout the fish development. It is important to highlight that the fluorescence intensities detected here may actually underestimate the amount of PS NPs in the developing zebrafish due to increased pigmentation and skull development that occurs during the time frame studied. The presence of PS NPs in the GI tract is in agreement with the findings of Skjolding et al. (2017) and Van Pomeroy et al. (2017), and PS NPs presence in the pericardium was also noted by Veneman et al. (2017). Most notably for this study, PS NPs were shown to localize in the pericardium only at 1 ppm and 10 ppm, but a significant decrease in the heart rate was observed even in larvae exposed to 0.1 ppm PS NPs. This suggests that even a low concentration of PS NPs is enough to observe cardiotoxicity. Moreover, the degree of fluorescence intensities decreased over time in all affected organs when the larvae were removed from the exposure media. The fluorescence observed in the 10 ppm PS NPs group was still significant after two days of depuration, but it returned to values similar to the control in lower concentrations. The high and constant fluorescence levels in the GI tract during depuration indicate at least two possible scenarios. The GI tract may be an important site for PS NPs excretion, which is

consistent with excretion route of carbon-based nanoparticles (Zhao et al., 2014). Alternatively, the clearance rate of PS NPs adsorbed within the intestinal tract is very slow, potentially impeding its function.

Significant PS NP fluorescence was also detected in the head, presumably the brain, of the zebrafish. This finding is consistent with previous studies that found PS NPs in brain tissues of fish exposed directly or through an aquatic food chain (Kashiwada, 2006; Mattsson et al., 2015a), suggesting that PS NPs are capable of penetrating the blood-brain barrier (Mattsson et al., 2017). PS NPs can translocate into red blood cells and are not membrane bound (Geiser et al., 2005), and therefore could enter the brain circulated via the blood. As the blood-brain barrier is established at approximately 72 hpf in zebrafish (Xie et al., 2010), and PS NPs were detected at significant fluorescence levels in the head of the embryos as soon as 24 hpf and 48 hpf, it could be expected that there is a larger PS NP uptake by the brain at early stages. After the blood-brain barrier is formed at 72 hpf, PS NPs fluorescence sharply decreases over time, suggesting that the faster clearance at later stages could be due to the formation of the blood-brain barrier. However, it has been previously shown that 53 nm PS NPs can pass through the blood-brain barrier (Mattsson et al., 2017). Thus, the role of the blood-barrier formation in the uptake of PS NPs should be examined in more details in future studies.

Given that PS NPs were shown to localize in the head (thus possibly the brain) as early as 24 hpf and throughout subsequent development, larval locomotor activity was evaluated to assess the potential of these nanoparticles to disrupt neural development and ultimately behavior. Zebrafish larvae exposed to PS NPs were hypoactive relative to controls over the course of an alternating light/dark test. This effect was driven by significantly reduced locomotor activity in the 1 ppm exposure group. Although this observation suggests that hypoactivity could be due to neurological differences rather than a mobility issue, the metabolic effects of PS NPs could not be excluded. While our OCR data suggests that there was no impact of PS NPs on mitochondrial function at 24 hpf, metabolic effects could occur later on due to a prolonged PS NPs exposure. Future studies should examine this issue on more details. It is noteworthy that decreased activity as well as altered feeding and shoaling behaviors have previously been shown in

adult Crucian Carp (*Carassius carassius*) exposed to PS NPs through an aquatic food chain (Mattsson et al. 2015a, Mattsson et al., 2017). It is possible that the PS NPs are triggering a specific targeting mechanism within the brain; however, further studies will be required to elucidate the precise mechanisms by which PS NPs exposure affects locomotor activity in early developmental stages. These findings are consistent with the hypoactivity observed by Chen et al. (2017), who suggested that PS NPs-induced hypoactivity is due to oxidative stress and reduced acetylcholinesterase activity. In an aquatic ecosystem, PS NPs-induced hypoactivity could reduce organismal fitness by affecting foraging or predator avoidance behaviors. While we show functional effects on heart and brain tissues herein, future studies will be necessary to determine whether there are functional effects on other tissue types where PS NPs accumulate.

The lack of mortality, morphological deformities, and normal mitochondrial metabolism suggest that PS NPs exposure would not result in acute lethal toxicity in wild fish populations. However, behavioral and cardiac effects could become pervasive throughout the fish populations. Specifically, both of these effects could reduce organismal ecological fitness, such as decreased ability to avoid predation, which could ultimately lead to decreased biodiversity in wild populations. It is also possible that the PS NPs could affect reproductive fitness or even cause transgenerational effects as PS NPs have been found to accumulate in the testes of zebrafish (Kashiwada, 2006). Future work should also examine how presence of nanoplastics can alter the effects of chemical, physical, and/or biological stressors, since aquatic organisms often face multiple stressors simultaneously.

In summary, the present study revealed that PS NPs can penetrate the zebrafish chorion and are taken up by the embryo. PS NPs initially localize to the yolk sac, but the particles migrate to the pericardium, head, pancreas, gallbladder, liver, and GI tract throughout development, even in the ppb range of exposure. Significant physiological effects of PS NPs exposure (e.g. bradycardia and hypoactivity) were quantified in tissues where the particles were shown to localize (e.g. pericardium and head, respectively), including at low exposure levels. Further, PS NPs exposures in the ppm range did not result in significant mortality or morphological deformities, suggesting that the observed toxic effects

were not secondary to teratogenesis. Together these data suggest that PS NPs may induce organ toxicity specific to their developmental distribution pattern.

Declaration of Interests

The authors have no competing interests.

Acknowledgements

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Figures

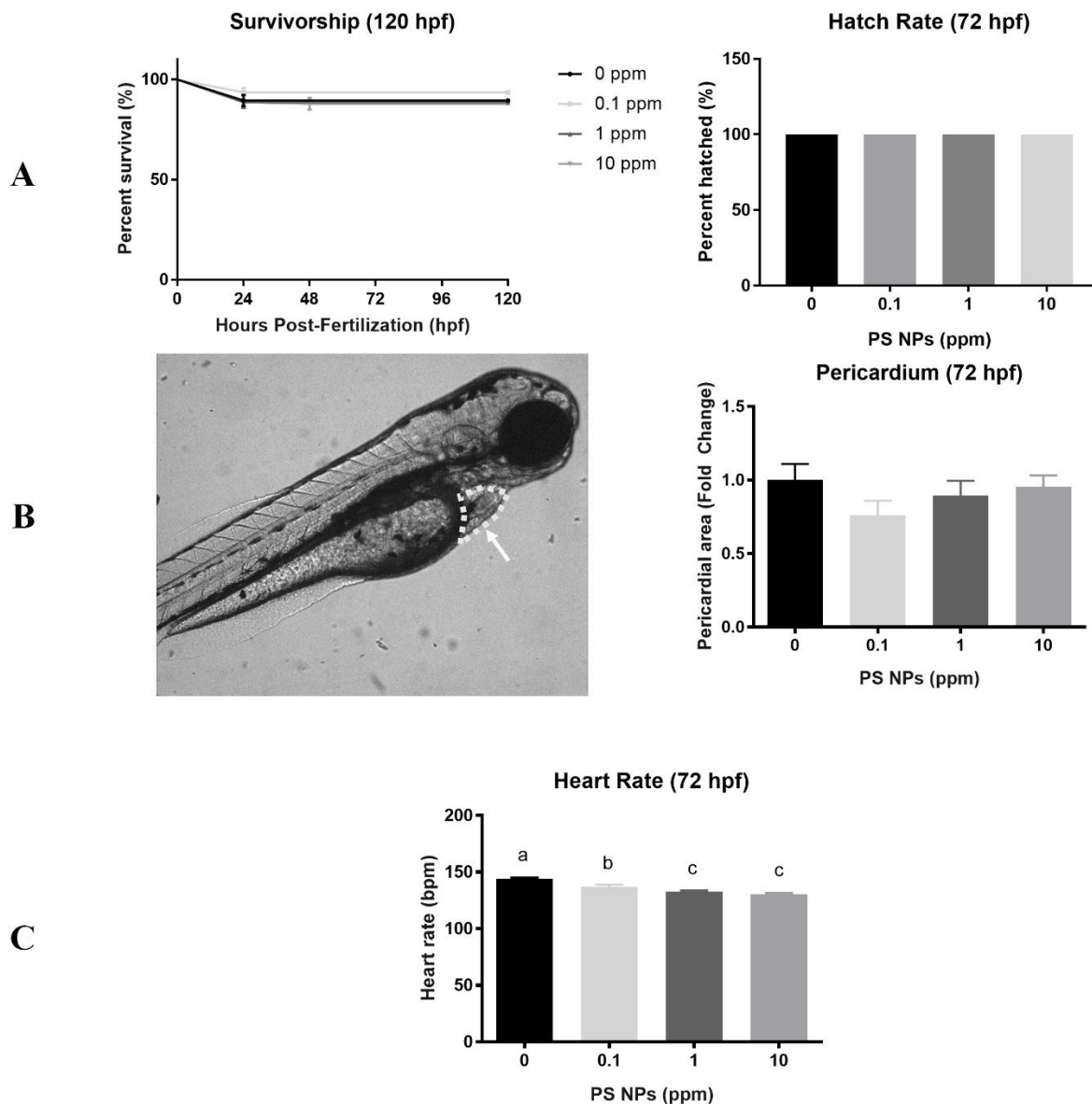


Figure 1. Survivorship, hatch rate, and cardiotoxicity in zebrafish larvae exposed to polystyrene nanoparticles (PS NPs).

(A) Mortality of larvae exposed to PS NPs. Survivorship from 24-120 hpf (n=124-240). Hatch rate was evaluated at 72 hpf (n=50). (B) Pericardial area expressed as fold change. The circled area that the arrow is pointing at in the image is representative of the pericardial area used (n=5). (C) Heart rate in beats per minute (bpm) (n=15). All data are presented as means \pm SEM. Significance was accepted if $p < 0.05$. Different letters denote statistical differences across treatments.

Figure 1 should be a 2-column fitting image.

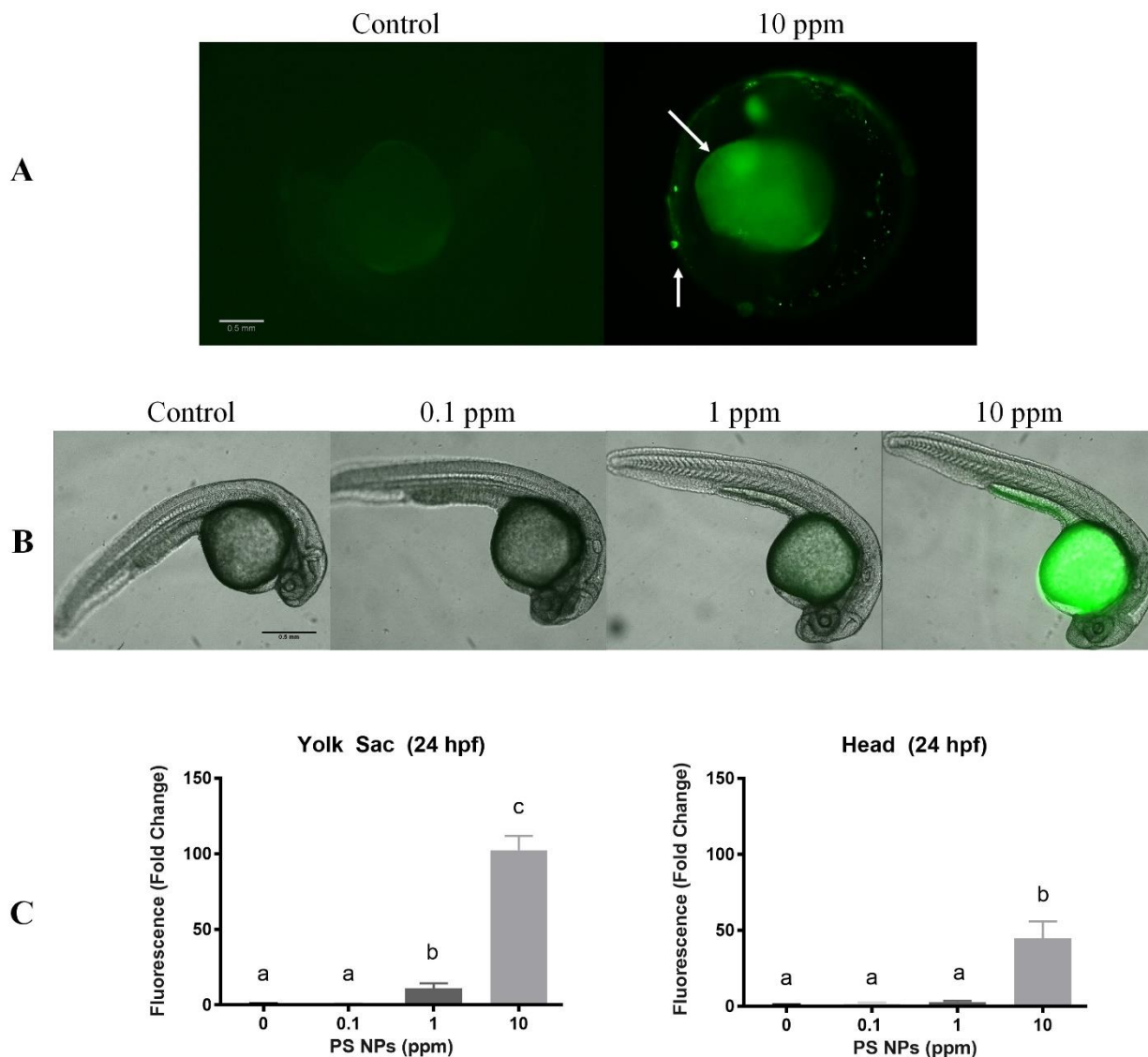


Figure 2. Polystyrene nanoparticles (PS NPs) fluorescence and distribution in 24 hpf zebrafish embryos

PS NPs fluorescence in zebrafish embryos at 24 hpf. Embryos were exposed with the chorion intact but were dechorionated prior to imaging (n=5). (A) Fluorescence in the chorionated zebrafish embryos. The arrows indicate PS NP aggregates on the surface of the chorion and fluorescence in the yolk sac. (B) Fluorescence in zebrafish embryos dechorionated prior to imaging to show PS NPs penetrated the chorion. (C) Fluorescence in the head and yolk sac of zebrafish embryos dechorionated prior to imaging. All fluorescence data are presented as fold change (means \pm SEM). Significance was accepted if $p < 0.05$. Different letters denote statistical differences across treatments

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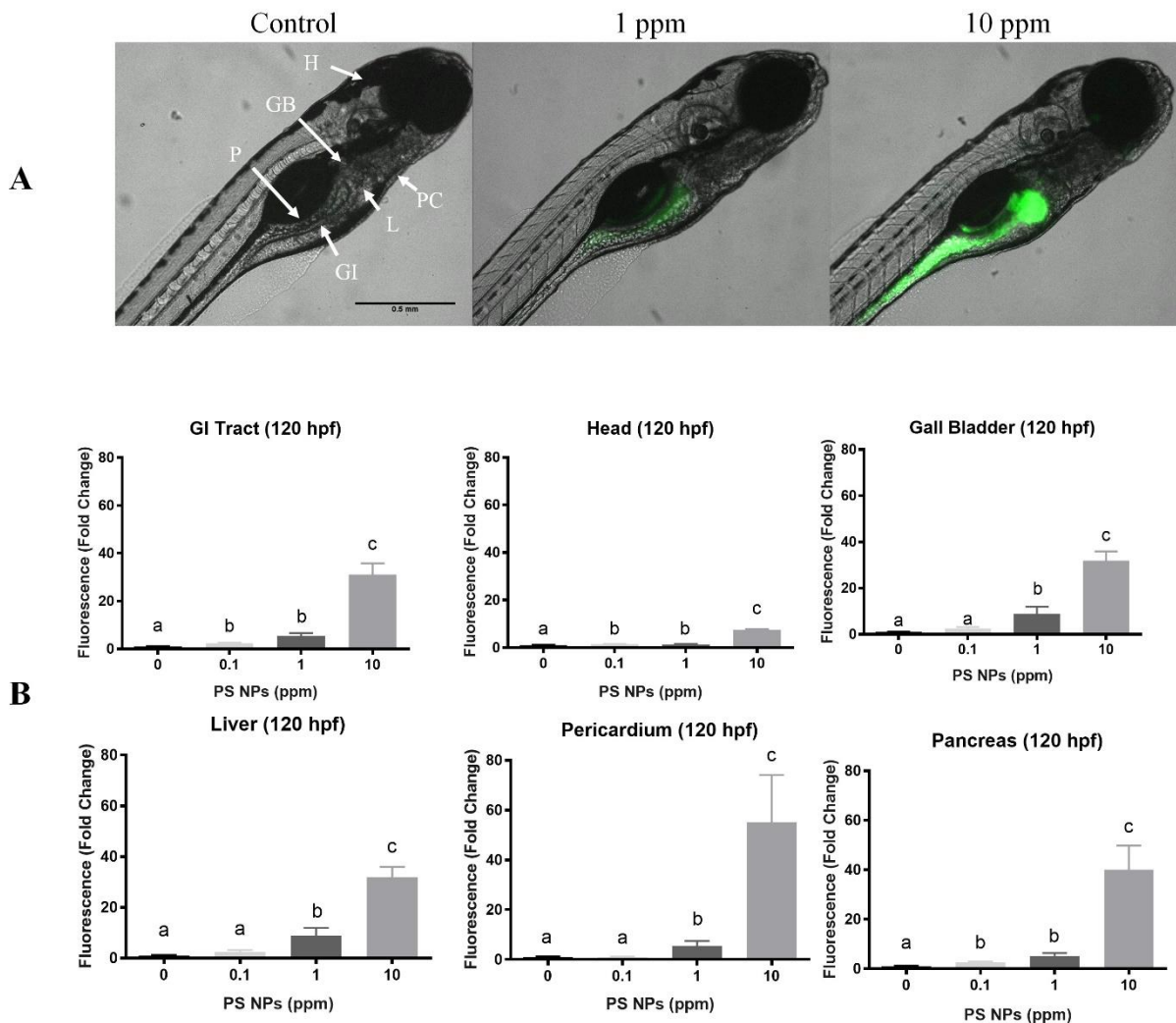


Figure 3. Polystyrene nanoparticles (PS NPs) fluorescence and distribution in 120 hpf zebrafish larvae

PS NPs fluorescence in zebrafish larvae at 120 hpf (n=5). (A) Fluorescence in the zebrafish larvae. The letters in the control image correspond to the various organs analyzed: (H) head, (GB) gall bladder, (P) pancreas, (PC) pericardium, (L) liver, (GI) gastrointestinal tract. (B) Fluorescence in the gastrointestinal tract, head, gall bladder, liver, pericardial area, and pancreas of zebrafish larvae presented as fold change (means \pm SEM). Significance was accepted if $p < 0.05$. Different letters denote statistical differences across treatments.

Figure 3 should be in color and a 2 column-fitting image.

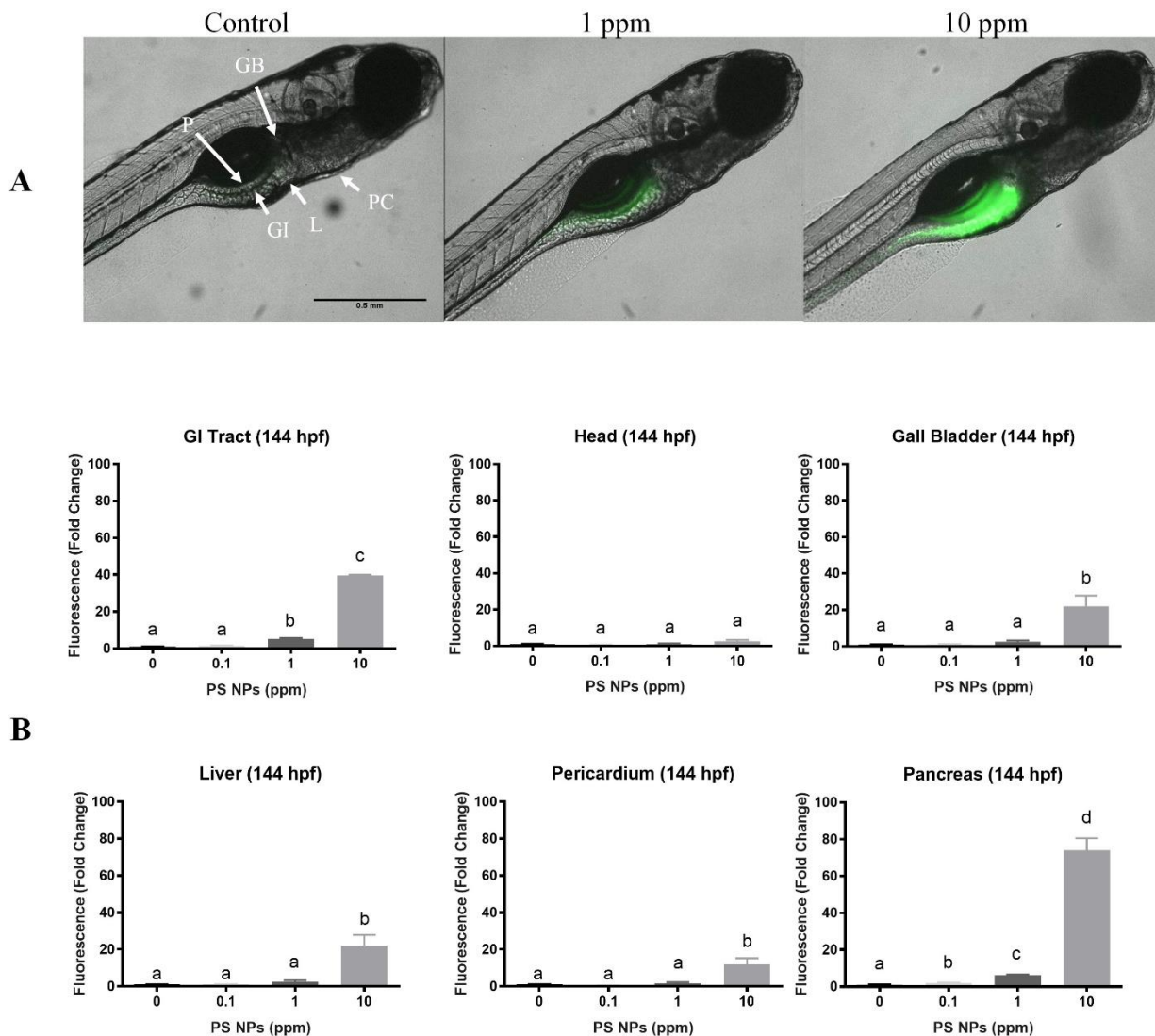


Figure 4. Polystyrene nanoparticles (PS NPs) fluorescence and distribution in 168 hpf zebrafish larvae

PS NPs fluorescence in zebrafish larvae at 168 hpf after 48 h of depuration (n=5). These images represent the last day of recovery. (A) Fluorescence in the zebrafish larvae. The letters in the control image correspond to the various organs analyzed: (H) head, (GB) gall bladder, (P) pancreas, (PC) pericardium, (L) liver, (GI) gastrointestinal tract. (B) Fluorescence of the gastrointestinal tract, head, gall bladder, liver, pericardial area, and pancreas of zebrafish larvae presented as fold change (means \pm SEM). Significance was accepted if $p < 0.05$. Different letters denote statistical differences across treatments.

Figure 4 should be in color and a 2-column fitting image.

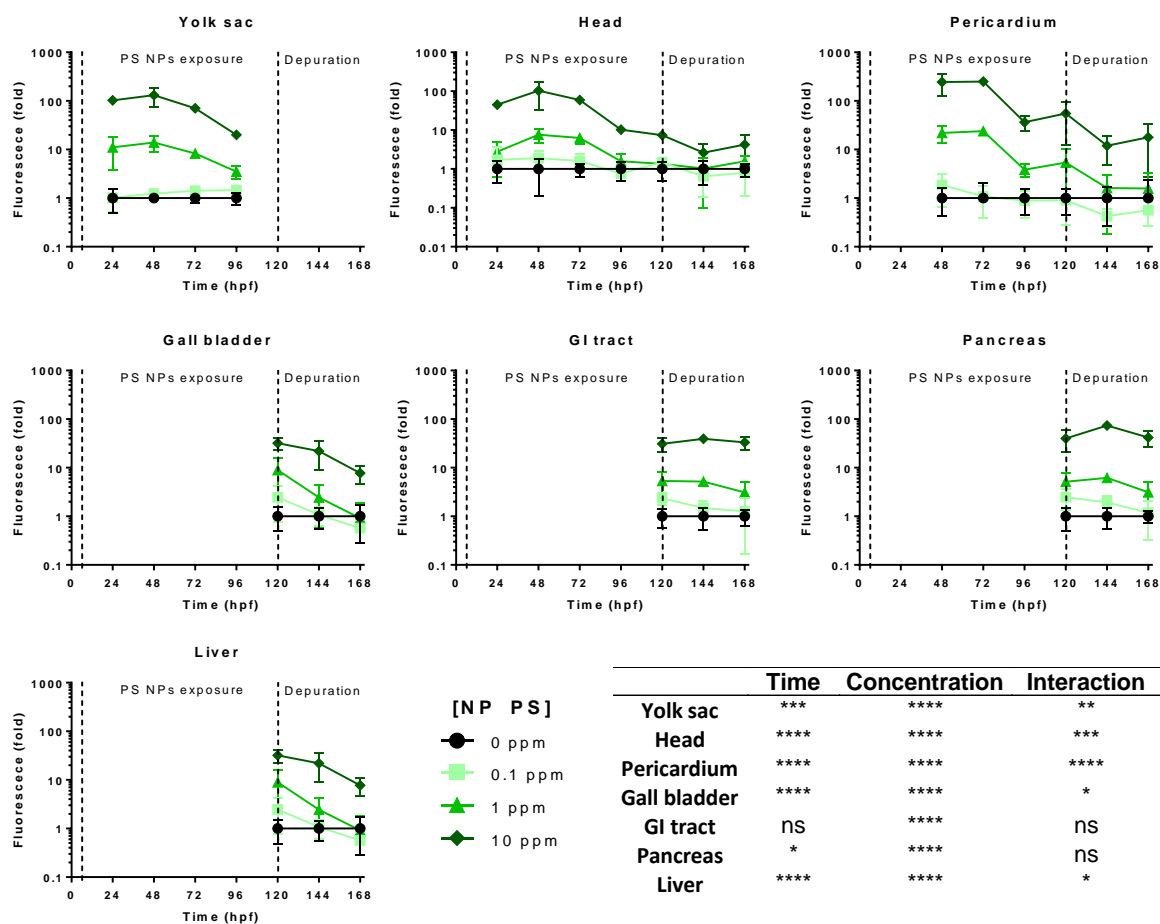


Fig 5. Polystyrene nanoparticles (PS NPs) fluorescence and distribution throughout the zebrafish development. Green fluorescence in different areas or organs of zebrafish embryos and larvae (n=5). Exposure to PS NPs started at 6 hpf and finished at 120 hpf. At this time point, PS NPs were removed and animals were allowed to depurate until 168 hpf. At 120 hpf, the yolk sac is resorbed, and the gall bladder, gastrointestinal (GI tract), pancreas, and liver are visible. Data are plotted against a logarithmic scale. Two-way ANOVA results are summarized in the bottom right panel, including the p-value for each factor (time of exposure and NP PSs concentration) and their interaction (* p<0.05, ** p<0.01, *** p<0.001, **** p<0.0001, ns p>0.05).

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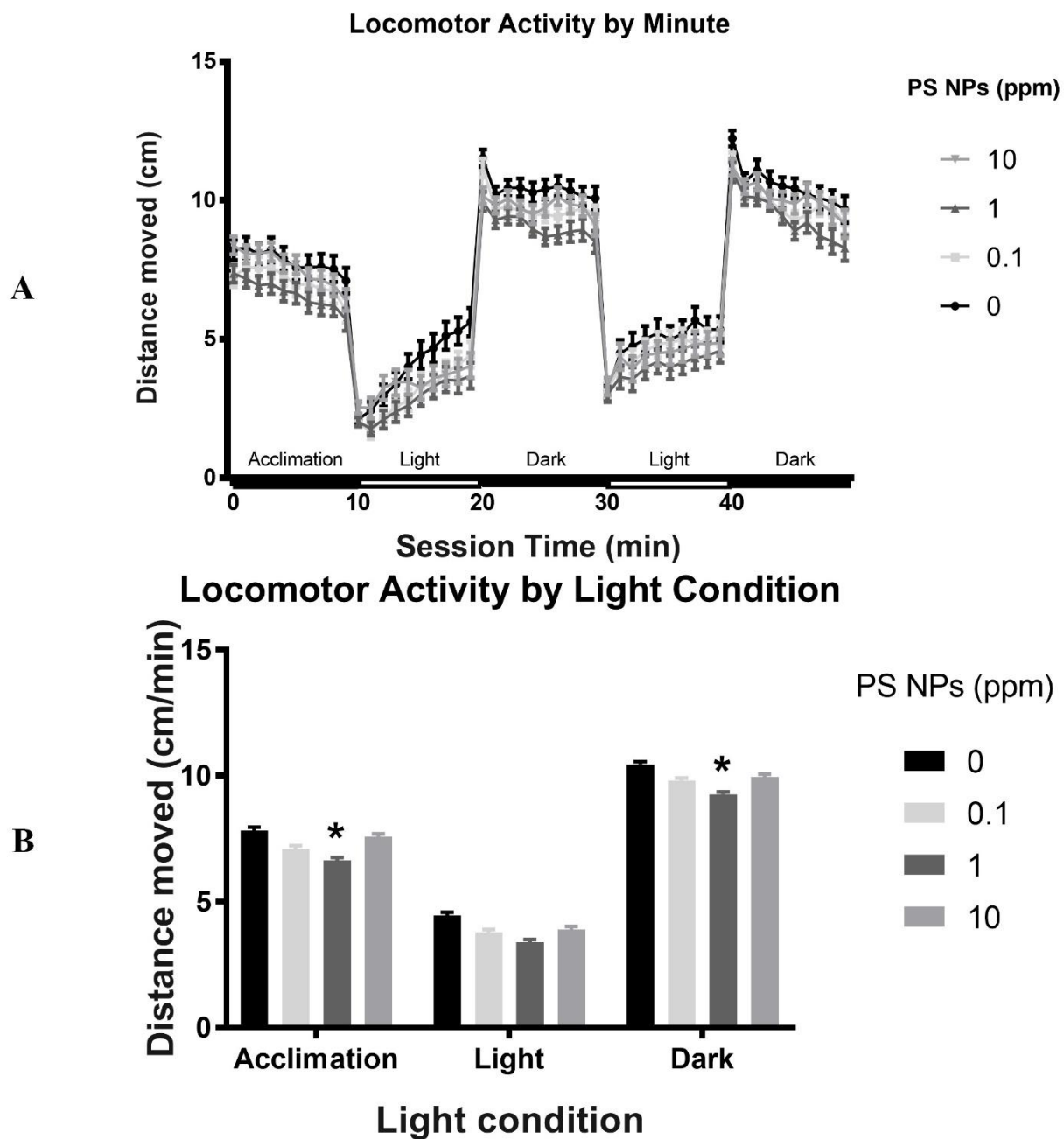


Fig 6. Behavioral toxicity in zebrafish larvae exposed to polystyrene nanoparticles (PS NPs). (A) Locomotor activity by light condition of larvae (144 hpf) exposed to PS NPs. The above plot represents average distance travelled per minute (cm/min) as a function of light condition (n=70-72). (B) Locomotor activity by light condition of larvae exposed to PS NPs. For both (A) & (B), data are presented as means \pm SEM. Significance was accepted if $p < 0.05$. * represents significant difference from control within the condition.

Figure 6 should a 2-column fitting image.

Supplemental Materials

Uptake, tissue distribution, and toxicity of polystyrene nanoparticles in developing zebrafish (*Danio rerio*)

Jordan A. Pitt^{1,2,*}, Jordan S. Kozal¹, Nishad Jayasundara^{1,3}, Andrey Massarsky¹, Rafael Trevisan¹, Nick Geitner⁴, Mark Wiesner⁴, Edward D. Levin⁵ & Richard T. Di Giulio¹

¹Nicholas School of the Environment, Duke University, Durham, NC 27708, USA.

²College of Environmental Science and Forestry, State University of New York, Syracuse, NY 13210, USA.

³School of Marine Sciences, University of Maine, Orono, Me 04469, USA

⁴Department of Civil and Environmental Engineering and the Center for the Environmental Implications of NanoTechnology, Durham, NC 27708, USA

⁵Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, NC 27710, USA

***Corresponding author:** jopitt@syr.edu

1. Methylene blue does not increase the toxicity of PS NPs

To evaluate the potential of PS NPs to act as carriers of methylene blue, thereby mediating the toxic effects that were observed with PS NPs exposure, we have compared the toxicity of 10 ppm PS NPs in media supplemented (0.00003%) and non-supplemented with methylene blue. The treatment groups included control, vehicle control (control veh; contains an equivalent concentration of SDS and sodium azide that is present in 10 ppm PS NPs group – 0.0001% and 0.00009%, respectively), and 10 ppm PS NPs. Exposure was carried out as described at section 2.4. The following endpoints were evaluated at 72 hpf: viability, hatching success, heart rate, and pericardial area. The results demonstrate that (1) methylene blue does not affect the toxicity of PS NPs, and (2) control and vehicle control group are not significantly different (Fig. S1). The results also confirm that exposure to 10 ppm PS NPs reduces the heart rate (Fig S1).

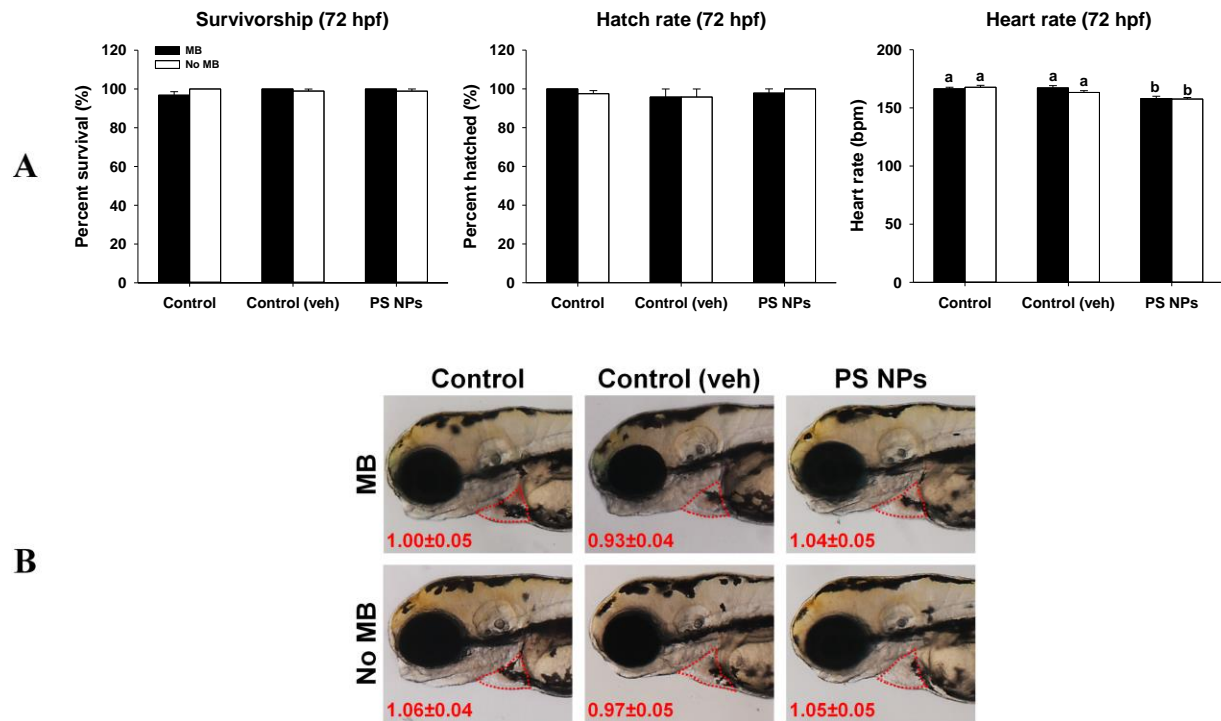


Figure S1. Toxicity of PS NPs in presence or absence of 0.00003% methylene blue (MB) in embryo medium. A. Survivorship, hatch rate, and heart rate were evaluated at 72 hpf in control, vehicle control (control veh; contains an equivalent concentration of SDS and sodium azide that is present in 10 ppm PS NPs group), and 10 ppm PS NPs-exposed larvae. Groups not sharing letters represent statistical differences assessed using a two-way ANOVA with a Fisher's LSD post hoc method ($p < 0.05$ was considered significant). B. Pericardial area (red dotted line) was assessed at 72 hpf and is indicated as fold change to control (with MB) in left bottom corner.

2. SDS and sodium azide do not increase toxicity and/or uptake of PS NPs

To evaluate the potential of SDS and sodium azide to affect the toxicity and uptake of PS NPs, we have compared the toxicity and uptake of 1 ppm PS NPs in presence of various concentrations of SDS and sodium azide. The treatment groups included control, vehicle control 1 (control veh 1; contains an equivalent concentration of SDS and sodium azide that is present in 1 ppm PS NPs group – 0.00001% and 0.000009%, respectively), vehicle control 2 (control veh 2; contains an equivalent concentration of SDS and sodium azide that is present in 10 ppm PS NPs group – 0.0001% and 0.00009%, respectively), 1 ppm PS NPs 1 (PS NPs 1; no additional SDS and sodium azide), and 1 ppm PS NPs 2 (PS NPs 2; contains an equivalent concentration of SDS and sodium azide that is present in 10 ppm PS NPs group). Exposure was carried out as described at section 2.4. The following endpoints were evaluated at 72 hpf: viability, hatching success, heart rate, pericardial area, and uptake. The results demonstrate that higher concentrations SDS and sodium azide do not increase the toxicity and uptake of PS NPs (Fig S2).

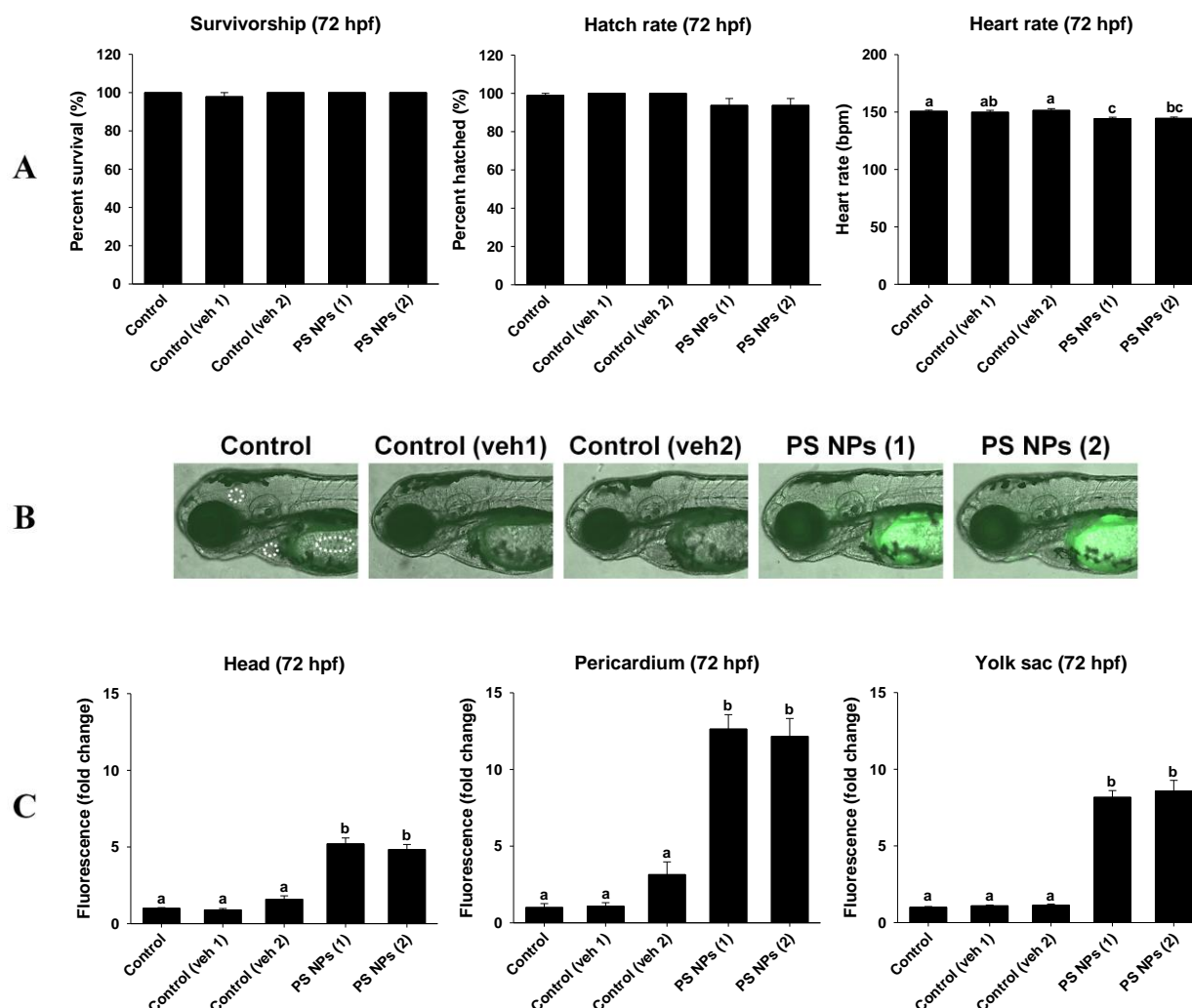


Figure S2. Toxicity and uptake of PS NPs in presence various concentrations of SDS and sodium azide. A. Survivorship, hatch rate, and heart rate were evaluated at 72 hpf in control, vehicle control 1 (control veh 1; contains an equivalent concentration of SDS and sodium azide that is present in 1 ppm PS NPs group), vehicle control 2 (control veh 2; contains an equivalent concentration of SDS and sodium azide that is present in 10 ppm PS NPs group), 1 ppm PS NPs 1 (PS NPs 1; no additional SDS and sodium azide), and 1 ppm PS NPs 2 (PS NPs 2; contains an equivalent concentration of SDS and sodium azide that is present in 10 ppm PS NPs group). B, C. Uptake of PS NPs was assessed at 72 hpf by quantifying fluorescence (fold change) in the head, pericardium, and yolk sac regions. Groups not sharing letters represent statistical differences assessed using a two-way ANOVA with a Fisher's LSD post hoc method ($p < 0.05$ was considered significant).

3. Fluorescence analysis of PS NPs uptake by zebrafish embryos and larvae.

To evaluate the uptake of PS NPs, analysis of fluorescence microscopy was carried out at different stages of the zebrafish development: 24 hpf (Fig. S3), 48 hpf (Fig. S4), and 144 hpf (Fig. S5). The treatment groups included control, 0.1 ppm PS NPs, 1ppm PS NPs, and 10 ppm PS NPs. Exposure was carried out as described at sections 2.4 and 2.6. Fluorescence was quantified at the indicated anatomical structures using ImageJ software.

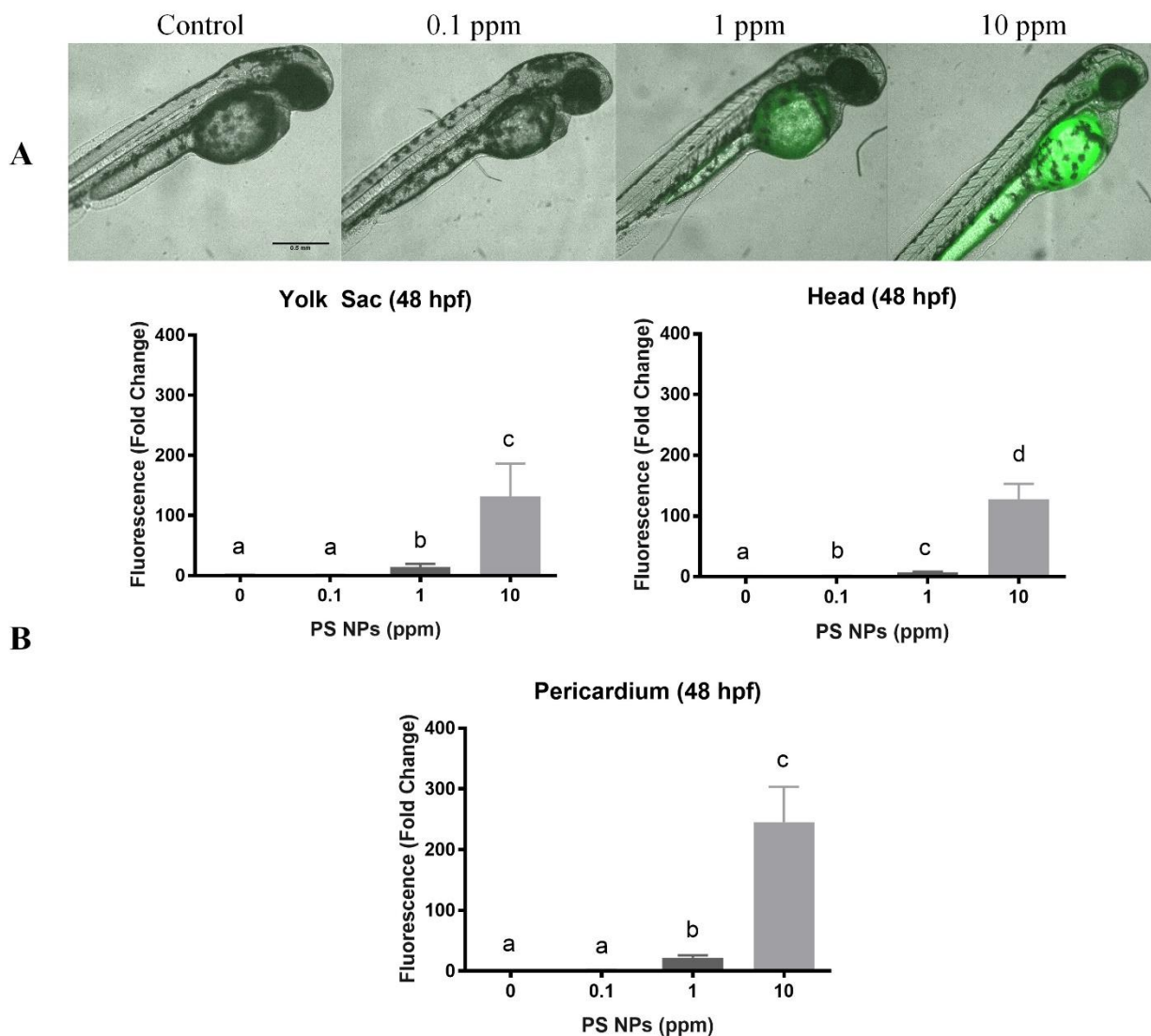


Figure S3. PS NPs fluorescence and distribution in 48 hpf zebrafish embryos

PS NPs fluorescence in zebrafish embryos at 48 hpf. (A) Fluorescence in the zebrafish larvae. (B) Fluorescence in the head, pericardial area, and yolk sac of zebrafish larvae expressed as fold change (means \pm SEM). Significance was accepted if $p < 0.05$. Different letters denote statistical differences across treatments.

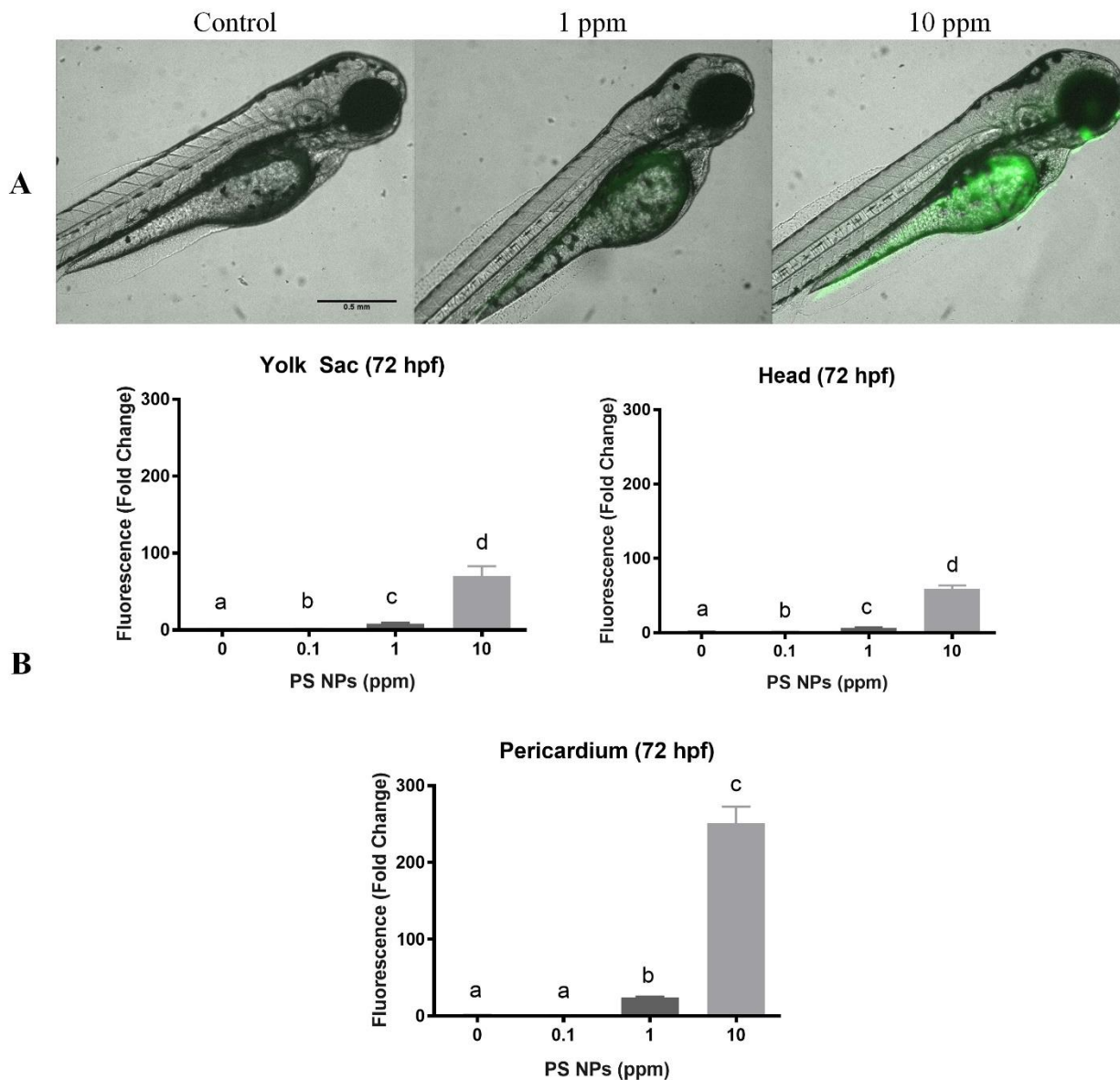


Figure S4. PS NPs fluorescence and distribution in 72 hpf zebrafish embryos

PS NPs fluorescence in zebrafish embryos at 72 hpf. (A) Fluorescence in the zebrafish larvae. (B) Fluorescence in the head, pericardial area, and yolk sac of zebrafish larvae expressed as fold change (means \pm SEM). Significance was accepted if $p < 0.05$. Different letters denote statistical differences across treatments.

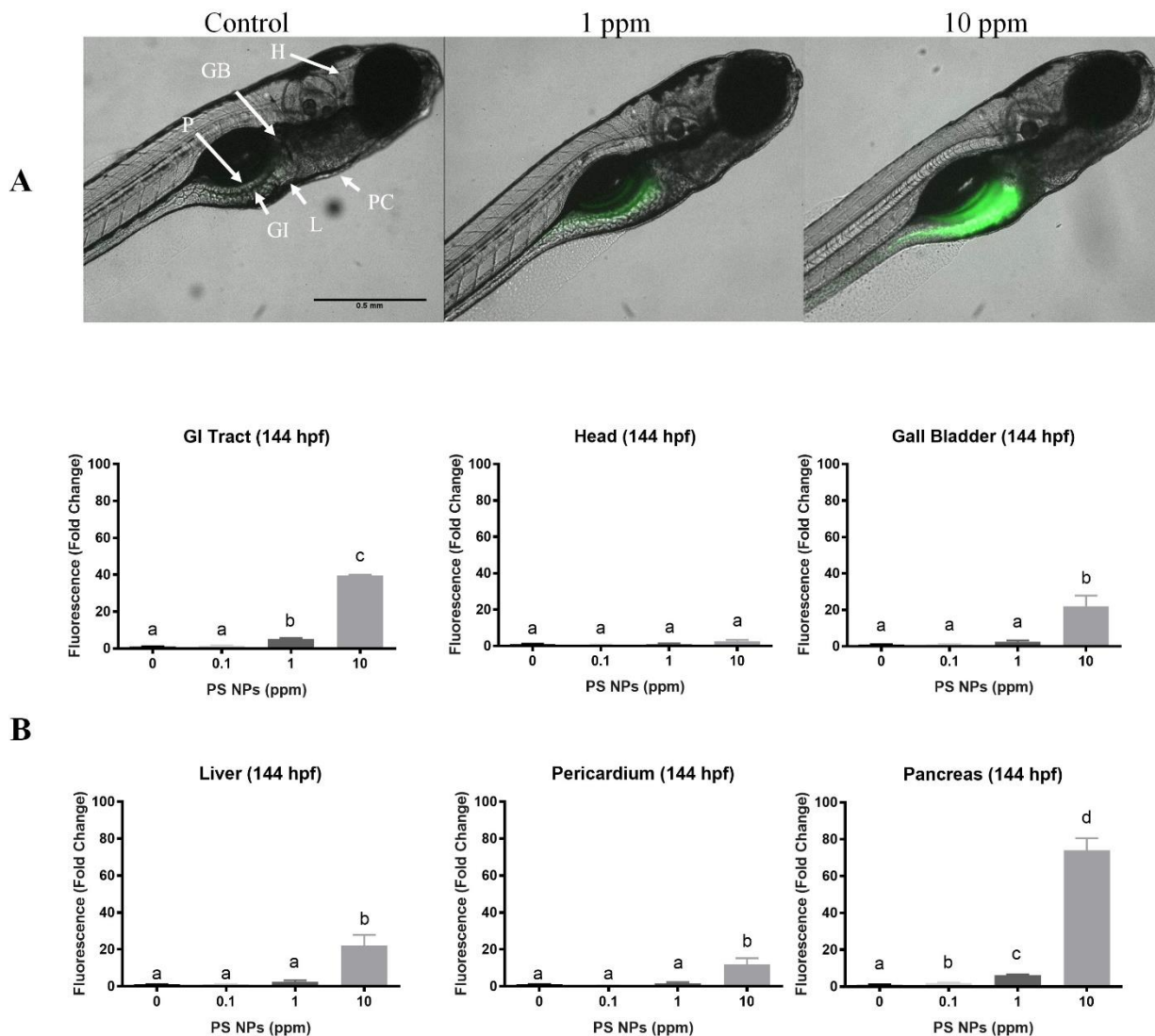


Figure S5. PS NPs fluorescence and distribution in 144 hpf zebrafish larvae

PS NPs fluorescence in zebrafish larvae at 144 hpf after 24 h of depuration (A) Fluorescence in the zebrafish larvae. The letters in the control image correspond to the various organs analyzed: (H) head, (GB) gall bladder, (P) pancreas, (PC) pericardium, (L) liver, (GI) gastrointestinal tract. (B) Fluorescence in the gastrointestinal tract, head, gall bladder, liver, pericardial area, and pancreas of zebrafish larvae presented as fold change (means \pm SEM). Significance was accepted if $p < 0.05$. Different letters denote statistical differences across treatments.

4. Effects of PS NPs on zebrafish larvae bioenergetics.

To evaluate the potential effects of PS NPs on zebrafish bioenergetics, oxygen consumption rate (OCR) and extracellular acidification rate (ECAR) were assessed using the XFe24 Extracellular Flux Analyzer in 144 hpf larvae. The treatment groups included control, 0.1 ppm PS NPs, 1 ppm PS NPs, and 10 ppm PS NPs. Exposure was carried out as described at sections 2.4 and 2.8.

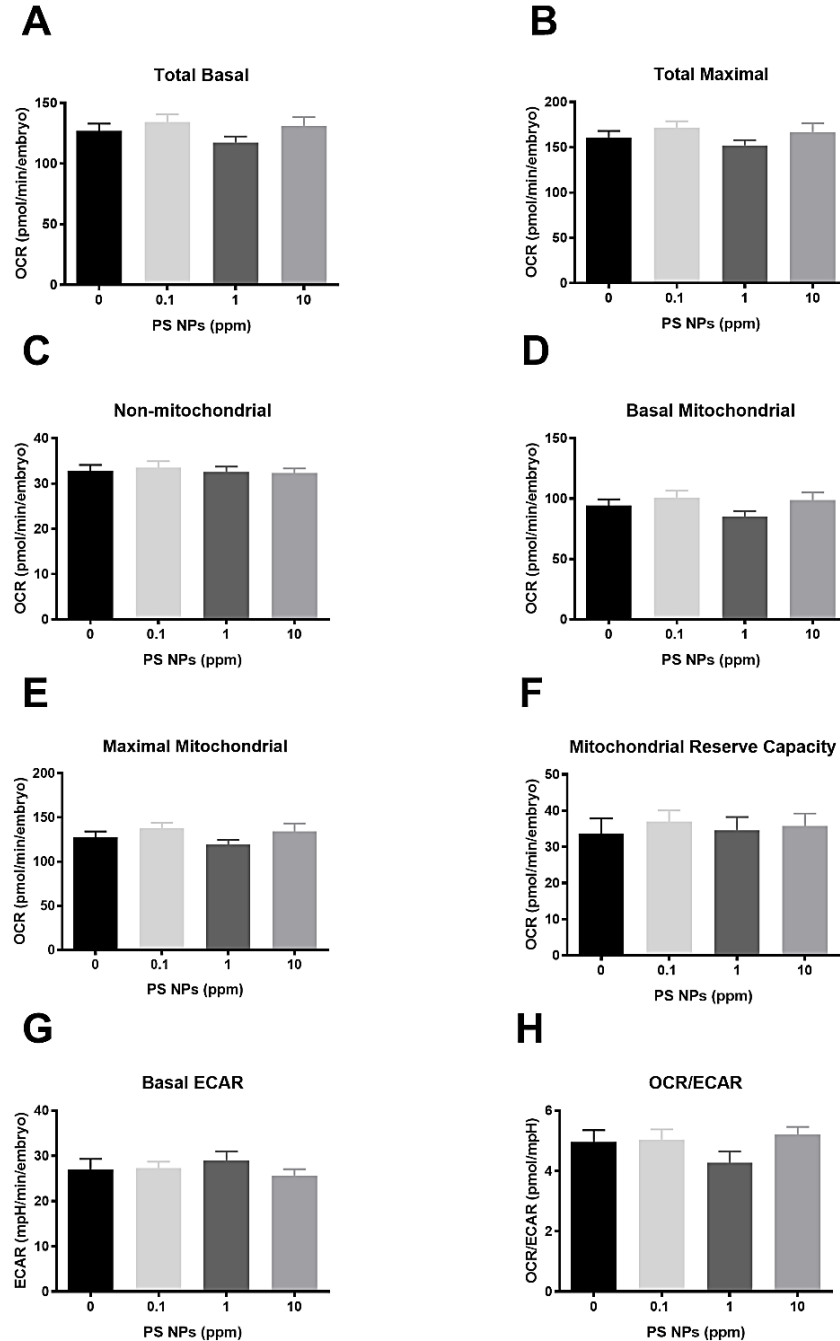


Figure S6. Mitochondrial function and metabolic partitioning in zebrafish embryos (24 hpf)

Respiration (oxygen consumption rate; OCR) due to various bioenergetic parameters measured *in vivo* in 24 hpf zebrafish embryos exposed to PS NPs (n=10). (A) Total basal respiration. (B) Total maximal respiration (in the presence of FCCP). (C) Non-mitochondrial respiration (in the presence of sodium azide). (D) Basal mitochondrial respiration (total basal – non-mitochondrial). (E) Maximal mitochondrial respiration (total maximal – non-mitochondrial). (F) Mitochondrial reserve capacity (total maximal – total basal). (G) Basal extracellular acidification rate (ECAR). (H) Ratio of basal OCR to ECAR. Data are presented as means \pm SEM. Significance was accepted if $p < 0.05$.

Maternal transfer of nanoplastics to offspring in Zebrafish (*Danio rerio*): a case study with nano polystyrene

Jordan A. Pitt^{1,2}, Rafael Trevisan¹, Andrey Massarsky¹, Jordan S. Kozal¹, Edward D. Levin³, & Richard T. Di Giulio¹

¹Nicholas School of the Environment, Duke University, Durham, NC 27708, USA.

²State University of New York, College of Environmental Science and Forestry, Syracuse, NY, 13210, USA.

³Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, NC 27710, USA

Abstract

Plastics are ubiquitous anthropogenic contaminants, including particles down to the nano-scale that are a growing concern in aquatic environments. The hazard of macro-plastics is well documented, but micro and nano-plastics remain relatively understudied. The current study examines the effects of a dietary exposure of adult fish on offspring, and whether or not oxidative stress and altered bioenergetics could play a role in polystyrene nanoparticles (PS NPs) toxicity using zebrafish (*Danio rerio*) as a model. Specifically, adult male and female zebrafish (F0 generation) were exposed to PS NPs via diet for a week. The fish were then bred to produce the F1 generation. Four F1 groups were generated: control (unexposed), maternal (exposed females), paternal (exposed males), and co-parental (exposed males and females). PS NPs dietary exposure of either parent did not significantly affect reproductive success. Assessment of tissues from F0 fish revealed that dietary exposure to PS NPs significantly reduced glutathione reductase activity, but did not affect mitochondrial bioenergetics parameters. Assessment of F1 embryos/larvae revealed that PS NPs were present in the yolk sac, gastrointestinal tract, liver, and pancreas of the maternally and co-parentally exposed F1 embryos/larvae. Bradycardia was also observed in embryos from maternal and co-parental exposure groups. In addition, the activity of glutathione reductase and the concentration of thiols were reduced in F1 embryos/larvae. Mitochondrial bioenergetics and behavioral activity were not affected in F1 larvae. This study suggests that PS NPs can be transferred via the female gametes from mothers to offspring and that oxidative stress may play a role in PS NPs toxicity.

Keywords: plastics, zebrafish, cross-generational, diet exposure, maternal transfer.

1. Introduction

The abundance of plastic in the oceans is estimated at 5.25 trillion particles and the number of particles continues to increase (Eriksen et al., 2014). Despite the increase in plastic pollution, nanoplastics (plastic particles with at least 1 dimension less than 100 nm) are a largely unknown danger (Andrady, 2011). The presence of nanoplastics is not yet quantified in marine systems due to limitations in analytical methods (Kolemans, 2015). However, nanoplastics are predicted to be present in the ocean due to the unexpectedly low levels of plastic debris smaller than 1 mm detected, it is predicted that the low levels are due to the particles undergoing fragmentation to a size below the detection limit (Cózar et al., 2014). These nanoplastics are thought to form primarily via photo- and physical degradation of larger plastic particles (Andrady, 2011).

The investigation of the toxicity of plastic particles is an emerging and rapidly developing field. The toxic potential of micro and nanoplastics [e.g., polystyrene nanoparticles (PS NPs)] is only starting to be understood. For example, blue mussels can accumulate up to 80 μm high-density polyethylene in their digestive gland, leading to the formation of granulocytomas and destabilization of lysosomal membranes (von Moos et al., 2012). PS NPs and carboxylated PS NPs can accumulate in marine organisms including sea urchin embryos (*Paracentrotus lividus*), brine shrimp (*Artemia fanciscana*), and water flea (*Daphnia magna*), indicating that PS NPs can biomagnify in the food web (Bergami et al., 2016; Della Torre et al., 2014; Mattsson et al., 2014).

Previous studies show that PS NPs accumulate in the brain of adult Crucian Carp (*Carassius carassius*), leading to behavioral changes (Mattsson et al. 2017). Behavioral alterations are also noted in zebrafish (*Danio rerio*) larvae, and are thought to be associated with oxidative stress and a reduction in acetylcholinesterase activity after PS NPs exposure (Chen et al., 2017). Additionally, PS NPs are reported to be capable of crossing the protective chorion of embryonic

Japanese medaka (*Oryzias latipes*) (Manabe et al., 2011). In zebrafish larvae, the PS NPs accumulate in the yolk sac before migrating to the gastro-intestinal tract, brain, and eye (Chen et al., 2017; Skjolding et al., 2017; van Pomerén et al., 2017).

Our previous study demonstrates that PS NPs are capable of penetrating the zebrafish chorion (Pitt et al., 2018). The particles accumulate in the yolk sac before migrating to the head, pericardium, gall bladder, gastrointestinal tract, liver, and pancreas (Pitt et al., 2018). Our previous studies also show several effects on embryos (e.g., bradycardia and hypoactivity), and in this study we aim to see if these effects persist via a parental or co-parental exposure (Pitt et al., 2018).

Accumulation of PS NPs in the embryo yolk sac suggests that egg yolk is a potential target for PS NPs accumulation in adult female fish. Accumulation of PS NPs in maternal gametes would create potential for transfer to the offspring and physiological effects. While these effects have not yet been examined in a vertebrate model, studies in non-vertebrate models support the potential for maternal exposure to PS NPs exerting adverse effects in offspring. For example, Zhao et al. (2017) reports that exposure of nematode *Caenorhabditis elegans* results in accumulation of PS NPs in the gonad. Lee et al. (2013) reports that exposure of copepod *Tigriopsis japonicas* to PS NPs decreases survival of exposed organisms as well as F1 offspring. Further, it is reported that *D. magna* and *Daphnia galeata* exposed to PS NPs show evidence of PS NPs penetrating the brood pouch and accumulating in the developing embryos (Brun et al., 2017; Cui et al., 2017). Based on these examples and the potential biomagnification of plastic in the food web, we hypothesize that maternal exposure may play a significant role in plastic exposure in various fish species.

To this end, the present study aims to assess the potential cross-generational bioaccumulation and toxicity of PS NPs in embryonic and early larval stages of F1 zebrafish following an F0 exposure. First, we predict that the PS NPs would accumulate in gonads

(especially in females) and be passed to the offspring. We also predict that exposure of adult zebrafish to PS NPs reduces the reproductive success and alters the antioxidant system and mitochondrial bioenergetics in various tissues. Further, we hypothesize that if PS NPs are passed on to offspring, then they would display similar distribution to that observed in embryos directly exposed to PS NPs [initial accumulation in the yolk sac followed by migration to the head, pericardium, gastrointestinal tract, gall bladder, liver, and pancreas (Pitt et al., 2018; Skjolding et al., 2017)]. Finally, we predict that the larvae would display signs of oxidative stress, bioenergetics effects, bradycardia, and behavioral changes (Chen et al., 2017, Pitt et al., 2018).

2. Materials and Methods

2.1. Materials

Fluorescent and non-fluorescent PS NPs (cat. #FSDG001 and #PS02002, respectively) were purchased from Bangs Laboratories, Inc. (Fishers, IN, USA). The fluorescent stock solution contained 1% (internally labeled with Dragon Green; ex./em. 480/520) PS NPs with a nominal mean diameter of 42 nm. The non-fluorescent stock solution contained 10% PS NPs also with a nominal mean diameter of 42 nm. Additionally, the stock solutions contained 0.1% sodium dodecyl sulfate (SDS) and 0.05-0.09% sodium azide. All other chemicals were purchased from Sigma-Aldrich unless otherwise specified.

2.2. PS NPs characterization and preparation

PS NPs were characterized using a dynamic light scattering (DLS) (Zetasizer Nano, Malvern Instruments Ltd., Malvern, UK). The hydrodynamic diameter and zeta potential of 5 ppm

PS NPs were assessed in 65 ppm artificial seawater (ASW; Instant Ocean, Blacksburg, VA, USA).

Prior to addition of PS NPs to zebrafish food (see section 2.3), the particles were centrifuged using a Vivaspin[®] 2mL Ultrafiltration Device (300,000 molecular weight cut-off, cat. #AA022) (Bangs Laboratories, Inc., Fishers, IN, USA) at 4,000 *g* for 10 min intervals to remove the sodium azide and SDS present within the solution. The PS NPs solution was then washed three times in DI water and filtered in the same manner. The PS NPs were then brought up to a final volume to reach 5% of the total solution by mass in DI water.

2.3. Diet preparation

Two diets were prepared: a control diet and PS NPs diet with fluorescent or non-fluorescent particles, depending on the experiment. To prepare the control diet, 90 mg of crushed Zeigler's Adult Zebrafish Complete Diet (Aquatic Habitats, Inc.), 45 mg of decapsulated brine shrimp egg, and 120 mg of gelatin (Carolina Biological Supply Company, Burlington, NC, USA) were added for every 1 mL of H₂O (Bisesi et al., 2015; Blickley et al., 2010; Blickely et al. 2014, Ennis, 2017). For the treated diet, PS NPs were added so that the final concentration of the particles would be 10% of the food by mass (the gelatin content was not considered part of the diet). This mixture was heated to 60°C and then vortexed. Enough food was prepared for a week of feeding based upon the average fish mass of each tank. Food was then transferred to vials in aliquots of the daily amount of food required for an average 1% of the total fish mass of each individual tank two times a day. The aliquots were stored in capped vials at 4°C.

2.4. Zebrafish husbandry

Laboratory reared AB wild-type (EkkWill Waterlife Resources; Ruskin, FL, USA) *D. rerio* were maintained in a recirculating AHAB system (Aquatic Habitats, Inc., Apopka, FL, USA) with a 14:10 h light/dark cycle. The water quality was sustained at 27-29°C and pH 7.0-8.0 in 65 ppm ASW. The fish were fed twice daily with brine shrimp (INVE aquaculture, Inc., Salt Lake City, UT, USA) in the morning and Zeigler's Adult Zebrafish Complete Diet (Aquatic Habitats, Inc.) in the afternoon.

2.5. Experimental set-up

Sexually mature, laboratory reared wild-type (EkkWill Waterlife Resources; Ruskin, FL) *D. rerio* (3-4 months with an average weight of 300 mg) were selected. The fish were acclimated to the control diet for a week prior to the exposure. Further, the fish were bred twice during the week prior to the start of exposure in order to acclimate them to breeding. Additionally, the fish were acclimated to the exposure tanks for two days. The fish were kept at a density of 6 fish of the same sex per 6 L of 65 ppm ASW with 2 tanks per exposure group for each sex. The tanks were kept at 28°C under constant aeration and physical and biological water filtration. The filters were turned off for 2 h during the feeding periods to prevent the loss of plastic particles. The day prior to the start of the exposure, the fish were bred (2 females: 2 males) to remove existing mature oocytes. During the 7 day exposure period, the fish were fed with the respective food diet (see section 2.3) twice per day (corresponding to 1% fish mass each time), once in the morning and once in the afternoon. The water was partially (50%) refreshed every other day.

At the end of the exposure, the parental (F0) fish were bred in the morning, and the F1 embryos were collected. All breeding crosses (2 females: 2 males) were set at 5 PM of the day before, and embryos were collected within 3 hours of spawning between 9 AM and 12 PM. The

embryos were kept in 30% Danieau's medium (in mM: 58 NaCl, 0.7 KCl, 0.4 MgSO₄, 0.6 Ca(NO₃)₂, 5 HEPES) and were stored in an incubator at 28°C. All zebrafish care and husbandry procedures were approved by the Duke University Institutional Animal Care and Use Committee (A139-16-06).

The F0 fish were bred between the different exposed conditions in order to obtain control (non-exposed), maternally (exposed females), paternally (exposed males), and co-parentally (exposed males and females) exposed F1 embryos. A total of 6 breeding pairs were used per non-fluorescent PS NPs experiment/cohort (6 males and 6 females each). A total of 2 breeding pairs were used per fluorescent PS NPs experiment/cohort (4 males and 4 females each). Following the breeding event, the fish were euthanized and the liver, gonads, muscle, and brain were dissected, collected into 1.5 mL Eppendorf tubes, flash frozen in liquid nitrogen, and stored at -80°C. The bioenergetics of the heart and gonads were measured with freshly dissected tissue samples (see section 2.10).

The experiment was repeated three times with the non-fluorescent PS NPs (total of 72 animals, 12 of each sex per experiment) and twice with the fluorescent PS NPs (total of 28 animals, 6-8 of each sex per experiment). The collected F1 embryos were screened at 6 h post-fertilization (hpf) for unfertilized eggs, which were then removed. At random 60 embryos per group (control, maternal, paternal, and co-parental) were transferred into two glass petri dishes (30 embryos/plate) at a density of 1 embryo/mL in 30% Danieau's for observations.

2.6. Assessments in F0 adults

2.6.1. Oxidative stress markers

The activities of antioxidant enzymes glutathione reductase (GR; EC 1.8.1.7), glutathione peroxidase (GPx; EC 1.11.1.9), and catalase (CAT; EC 1.11.1.6) were assessed in brain, liver, muscle, ovaries, and testes of adult F0 zebrafish. The tissues were homogenized in ice-cold 50 mM potassium phosphate buffer pH 7.0 containing 0.5 mM EDTA and protease inhibitor cocktail, using a probe sonicator. The homogenates were centrifuged at 3,000 g for 15 min and the resulting supernatants were transferred to fresh 0.5 mL Eppendorf tubes. GR activity was measured by following the consumption of NADPH (0.2 mM) at 340 nm in the presence of oxidized glutathione (GSSG, 1 mM) (Carbelrg and Mannervick, 1985). GPx activity was measured by following the consumption of NADPH (0.2 mM) at 340 nm, but in presence of 1 mM cumene hydroperoxide, 1 mM reduced glutathione (GSH), and 0.2 U/ml baker's yeast GR (Wendel, 1981). CAT activity was measured by following the degradation of 10 mM H₂O₂ at 240 nm in presence of 0.01% Triton X-100 (Aebi, 1984). Spectramax M5 plate reader (Molecular Devices, CA, USA) was used to measure the absorbance at aforementioned wavelengths and were carried out in 50 mM potassium phosphate buffer pH 7.0 containing 0.5 mM EDTA. Enzyme activities were normalized to protein concentration in the supernatant, which was measured using the Pierce BCA protein content kit (Thermo Fisher Scientific, MA, USA). The enzyme activities were further normalized to the control and expressed as fold change.

2.6.2. Bioenergetics

The oxygen consumption rate (OCR) in heart and gonads of F0 adult fish was assessed *ex-vivo* using the XFe24 Extracellular Flux Analyzer (Agilent Instruments, CA, USA) according to Jayasundara et al. (2015). The fish were euthanized in ice-cold water, and the heart and gonads were extracted and then rinsed with Ringer's solution. For the ovaries only about 10 mg of tissue

were used, while the other tissues were used in full (0.6 mg heart tissue and 0.9 mg testes tissue). The tissues were placed into 525 μ L Ringer's in separate wells of an XFe24 islet capture microplate (Agilent Instruments, CA, USA) (4 blanks were used per plate with 2-4 tissues each type/group/plate). Each measurement cycle consisted of 2 min intervals of mixing, waiting, and measurement periods. Basal OCR was measured over 8 cycles, prior to the injection of pharmacological agents: i) the mitochondrial uncoupler carbonyl cyanide 4-(trifluoromethoxy) phenylhydrazone (FCCP) (2.5 μ M; maximal OCR), ii) a mixture of the cytochrome c reductase (complex III) inhibitor, antimycin A, and the NADH dehydrogenase inhibitor, rotenone (44 μ M each; non-mitochondrial OCR). The OCR values were normalized by the weight of the tissue used. The OCR values were used to calculate total mitochondrial respiration (Basal – Antimycin A/Rotenone), maximal mitochondrial respiration (FCCP – Antimycin A/Rotenone), and spare capacity (FCCP – Basal).

2.6.3. Reproductive success

The effect of dietary PS NPs exposure on reproductive success was estimated by counting the total number of eggs produced and their fertilization rate after each breeding event.

2.7. Assessments in F1 embryos/larvae

2.7.1. General physiology

The F1 embryos and larvae parentally-exposed to non-fluorescent PS NPs were screened daily for mortality until 96 hpf, with dead individuals being removed (n=4 plates, with 2 plates of 30 embryos per cohort and 2 cohorts). Heart rate was measured at 48 hpf from 10 randomly selected larvae (n=20 embryos over 2 cohorts, 5 from each petri dish). Larval deformities were

visually assessed from 24-96 hpf using a dissecting microscope to examine each larvae (n=4 plates, with 2 plates of 30 embryos per cohort and 2 cohorts).

2.7.2. Distribution of PS NPs

The F1 embryos and larvae parentally-exposed to fluorescent PS NPs were imaged using a Zeiss Axioskop (Carl Zeiss, Oberkochen, Germany) fluorescence microscope at 24 h intervals from 24 hpf to 144 hpf. At each time point, 3 randomly selected individuals from each group were imaged (n=6 embryos or larvae over two cohorts). At 24 hpf the zebrafish were imaged with intact chorions, and at 48 hpf larvae that had already hatched were selected for imaging. Fluorescence images were captured using a 75W/2 xenon short-arc lamp, a Photometric CoolSNAPfx monochrome CCD camera (Roper Scientific, Tuscon, AR, USA), 480/30 nm (em) and 535/40 nm (ex) filters with a 1 s camera exposure. Fluorescence intensity was analyzed using ImageJ software and the values were normalized to the control fluorescence and expressed as fold change.

2.7.3. Oxidative stress markers

2.7.3.1. Thiol levels

F1 zebrafish larvae (96 hpf) were transferred into a 16 well plate and incubated in presence of 300 μ M monobromobimane (mBrB) in the dark at room temperature for four hours (n=16 larvae across 2 cohorts, 100 μ L of mBrB per larva). After incubation, the larvae were rinsed three times with fresh 30% Danieau's, and individually transferred into a 384-well plate with 100 μ L of Danieau's/well. The fluorescence levels were measured using a Spectramax M5 plate reader (Molecular Devices, CA, USA) with 340/10 nm (ex) and 520 nm (em) filters. The fluorescence

levels were subtracted from a blank (Danieau's only), normalized to the control, and expressed as fold change.

2.7.3.2. Redox state

F1 zebrafish larvae (96 hpf) were transferred into a 16 well plate and incubated in presence of 200 µg/mL resazurin in the dark at room temperature for four hours (n=14-16 larvae across 2 cohorts, 100 µL resazurin per larva). After incubation, the larvae were rinsed three times with fresh 30% Danieau's, and individually transferred into a 384-well solid plate with 100 µL of pure methanol for resorufin extraction. The larvae were incubated in methanol for 30 min. Subsequently, fluorescence was measured using a Spectramax M5 plate reader with 550/10 nm (ex) and 590 nm (em) filters. The fluorescence levels were subtracted from a blank (Danieau's only), normalized to the control, and expressed as fold change.

2.7.3.3. Antioxidant enzymes

The activities of antioxidant enzymes GR, GPx, and CAT were assessed in 96 hpf zebrafish larvae. The larvae were homogenized in ice-cold 50 mM potassium phosphate buffer (pH 7.0) containing 0.5 mM EDTA and protease inhibitor cocktail. The homogenates were centrifuged the resulting supernatants were used to assess enzyme activities following the methods described above.

2.7.4. Bioenergetics

The OCR was assessed in 24 hpf F1 zebrafish embryos using the XFe24 Extracellular Flux Analyzer (Agilent Instruments, CA, USA) according to Stackley et al. (2011). For this assessment,

embryos were individually staged in a 24 well plate with 700 μ L of 66 ppm ASW (5 larvae per group with 4 blank wells). OCR measurements were performed as mentioned above, but with cytochrome c oxidase inhibitor sodium azide (6.25 mM NaN_3) instead of antimycin A/rotenone. The OCR values were used to calculate total mitochondrial respiration (Basal – NaN_3), maximal mitochondrial respiration (FCCP – NaN_3), and spare capacity (FCCP – Basal).

2.7.5. Larval behavior

At 96 hpf, F1 zebrafish larvae were transferred into beakers with 200 mL of 30% Danieau's. The larvae were then transported to the behavioral facility, where they were kept on a 14:10 h light/dark cycle at 28°C. At 144 hpf the larvae were individually transferred into a 96 well plate (n=36-48 larvae over 2 cohorts, with 12-24 larvae per cohort). The larvae were allowed to acclimate in the dark for one hour prior to being transferred to a DanioVision™ observation chamber (Noldus Inc., Wageningen, The Netherlands) for an alternating light/dark test where larval locomotor activity is recorded (Massarsky et al., 2015; Bailey et al., 2016). During the 50 minute test, the first 10 minute period is habituation in the dark, and then the next 40 minutes are broken up into two cycles of 10 minute alternating light/dark periods. Video data were recorded at a sample rate of 30 frames/second via a high-speed infrared camera. The EthoVisionXT® software (Noldus, Wageningen, The Netherlands) was used to calculate total distance moved for each individual larvae throughout the test.

2.8. Statistical analysis

The results are presented as mean \pm standard error of the mean. For F1 embryo and larval endpoints, one-way Analysis of Variance (ANOVA) with a post hoc Tukey method was used to assess statistical differences between treatment groups. Two-way ANOVA with a post hoc Tukey

method was used to analyze cohort effect and locomotor activity. The F0 adult enzyme analysis was completed with an unpaired t-test. These analyses were conducted using Graph Pad Prism 6.0 (San Diego, CA, USA). For all endpoints, $p < 0.05$ was considered statistically significant.

3. Results

3.1. PS NPs characterization

The non-fluorescent PS NPs (5 ppm in ASW medium) had a mean hydrodynamic diameter of 30.67 ± 8.97 nm. The zeta potential was -24.7 ± 2.93 mV. The fluorescent PS NPs (5 ppm in ASW medium) had a mean hydrodynamic diameter of 34.5 ± 10.8 nm. The zeta potential for these particles was -21.1 ± 2.47 mV. Both values indicate a low aggregation behavior for both types of PS NPs.

3.2. Assessments in F0 adults

3.2.1. Oxidative stress markers

GR activity was significantly lower in brain and muscle of exposed females and muscle of exposed males (Fig. 1). GPx activity was elevated in the brain of exposed females, but no changes were detected in males (Fig. 1). CAT activity was not significantly affected by the dietary exposure to PS NPs in any of the tissues in males or females (Fig. 1).

3.2.2. Bioenergetics

The *ex vivo* bioenergetics analysis of heart and gonad tissues did not reveal statistically significant changes in any of the analyzed parameters (Fig. 2). However, a marginal effect of PS NPs can be seen in female gonads, such that all OCR values (basal, maximum, non-mitochondrial,

basal mitochondrial, and mitochondrial spare capacity) were slightly higher than the control group ($p = 0.08-0.12$, Fig. 2).

3.2.3. Reproductive success

There was no statistically significant effect of PS NPs exposure on the number of eggs spawned (Fig. 3A). The fertilization rate was also not significantly different (Fig. 3B).

3.3. Assessments in F1 embryos/larvae

3.3.1. General physiology

Mortality was not significantly different between the different exposure groups in the F1 embryos throughout embryonic and larval development at any of the measured time points (24- 96 hpf) (Fig. 3C). Heart rate was significantly decreased in F1 larvae from the maternal and co-parental exposure groups with the paternal group trending relative to the control (Fig. 3D). No deformities were noted in parentally-exposed embryos/larvae; however, the swim bladders of larvae that were exposed to PS NPs co-parentally were uninflated at 144 hpf (Fig. 3E).

3.3.2. Distribution of PS NPs

Green fluorescence was used to semi-quantitatively evaluate distribution of PS NPs in F1 zebrafish embryos and larvae from 24 hpf to 120 hpf. At 24 hpf, fluorescence was observed in the yolk sac of F1 embryos (Fig. 4). The observed fluorescence was significantly higher in the co-parental exposure group (7 fold) and elevated but not significantly in the maternal exposure group (4 fold) relative to the controls (Fig. 4). At 48 and 72 hpf, the same fluorescence pattern was observed in the yolk sac (Fig. 4).

By 120 hpf, the larvae have a fully functional and developed GI tract, liver, pancreas, and gall bladder. The co-parentally exposed F1 larvae at this time point had a significant amount of fluorescence in the liver and GI tract (Fig. 5). Additionally, the maternally exposed F1 larvae had a significant amount of fluorescence in the GI tract and pancreas (Fig. 5). The gall bladder, while not significant, did have a variable amount of increased fluorescence in the co-parental and maternal exposure groups (Fig. 5).

3.3.3. Oxidative stress markers

Fluorescence of monobromobimane (MBrB) was used to qualitatively assess the differences in total thiol levels (reduced forms of protein and non-protein thiols) in F1 96 hpf larval zebrafish relative to controls. The fluorescence was significantly reduced in both the maternal and co-parental exposure groups (Fig. 6).

The redox sensor resazurin was used to quantify the overall redox state of the F1 96 hpf larvae. No indicator of major oxidation events was found in the larvae due to no change in fluorescence relative to the controls for any of the exposure groups (Fig. 6).

Three antioxidant enzymes' activities were quantified in the F1 96 hpf larvae. GR activity was significantly reduced in the F1 co-parental exposure group compared to the control (Fig. 6). Activities of CAT and GPx were not different in any of the exposure groups for the F1 generation (Fig. 6).

3.3.4. Bioenergetics

In vivo bioenergetics analysis in F1 24 hpf embryos revealed no statistically significant differences in any of the OCR parameters (Fig. S1).

3.3.5. Larval behavior

Larval locomotor activity was monitored over the course of an alternating light/dark test at 144 hpf. There appeared to be no significant effect on larval locomotor activity for any of the F1 larvae (Fig. S1).

4. Discussion

The potential exposure routes and effects of nanoplastics on vertebrate organisms is largely unknown. Our previous study has shown that PS NPs are capable of penetrating the zebrafish chorion, and accumulating in the yolk sac before migrating to the head, pericardium, liver, gastrointestinal tract, gall bladder, and pancreas (Pitt et al., 2018). The presence of the PS NPs then induced bradycardia and hypoactivity in exposed larva (Pitt et al., 2018). These results from a waterborne exposure led us to look at what differences would result from a dietary exposure. To that end, several aspects were assessed in both F0 adults and F1 embryos/larvae. Additionally, this study addresses the effects of nanoplastics on aquatic species using a maternal transfer exposure as an environmentally relevant exposure pathway. Adult zebrafish were fed with a diet containing PS NPs (10% of the food by mass). The F0 tissues as well as their offspring were analyzed for markers of oxidative stress, mitochondrial bioenergetics, embryonic development and larval behavior. The transfer of PS NPs from F0 to F1 was addressed by using fluorescent PS NPs and fluorescence microscopy. The following sections detail the effects on enzymes related to oxidative stress in the F0 generation in addition to examining the bioenergetics effects. Following this the physiological effects, distribution, oxidative stress biomarkers, and bioenergetics of the F1 generation are discussed.

First we show that dietary exposure to PS NPs cause the modulation of the antioxidant system in the F0 generation. This was evidenced by the reduction of GR activity in the brain of females and muscle of both females and males and the increased GPx activity in the brain of females. Both GPx and GR are related to glutathione metabolism, which is an important cellular process responsible, in part, for the detoxification of peroxides and electrophilic agents. The imbalance of reactive oxygen species (ROS) production and detoxification in favor of the former can result in higher concentrations of ROS, such as hydrogen peroxide and organic peroxides, leading to oxidative stress and potentially cellular damage, and apoptosis (Sies and Jones, 2007). Although the interpretation of oxidative stress based upon antioxidant activities data alone provides a limited insight about the oxidative potential of PS NPs, the effects on GPx and GR activities in the brain are in agreement with studies that have reported accumulation of nanoplastics in brain of adult crucian carp (Mattsson et al., 2017). In addition, micro- and nano-sized polystyrene particles were shown to have pro-oxidant properties, leading to decreased levels of reduced glutathione in zebrafish larvae (Chen et al., 2017), and increased ROS levels and antioxidant enzyme activities in copepods *Paracyclopsina nana* (Jeong et al., 2016, 2017). Our data provide further support that PS NPs potentially increase ROS and interfere with the antioxidant system of aquatic species, which can potentially lead to further cellular and physiological impairments and reduced capability to respond to additional environmental stressors.

Moreover, ROS production and impairment of the antioxidant system could lead to mitochondrial dysfunction (Dranka et al., 2011), which has been reported for several environmental toxicants (Jayasundara, 2017). In this study, the mitochondrial metabolic profile was not altered in the heart or gonads of F0 adults. However, the ovary tissues were trending towards having a significantly increased OCR for all parameters except for the mitochondrial

reserve capacity. It is still unknown whether nano or microplastics are able to cause significant changes in mitochondrial function, and future studies should examine in more detail the potential pro-oxidant properties of PS NPs and the potential adverse effects on mitochondrial function.

The main aim of this study was investigate whether parental exposure to PS NPs can have adverse impacts on the offspring. We demonstrate that PS NPs were present in the yolk sac of embryos maternally or co-parentally exposed F1 embryos/larvae. To the best of our knowledge, this is the first study to demonstrate that PS NPs are capable of having a cross-generational effect in a vertebrate species, which has significant ecological implications. The PS NPs were seen to accumulate first in the yolk sac as early as 24 hpf, which was also observed in our previous study (Pitt et al., 2018). The lipid heavy composition of the vitellogenin which PS NPs have previously been shown to interact with, is the likely target of PS NPs accumulation in F0 females (Rossi et al., 2013). These results also agree with Cui et al. (2017), who reported that PS NPs were passed down through generations in crustaceans with the PS NPs binding to the lipophilic areas in both F0 and F1 generations. It is unclear how maternal transfer of PS NPs is mediated, but studies with other nanoparticles suggest that certain nanoparticles have high affinity for plasma proteins, including lipid transport proteins vitellogenin and zona pellucida (Gao et al., 2017). These transfer proteins could facilitate the transfer of PS NPs to the oocytes, and ultimately, the embryo yolk sac. This could explain why only maternally-exposed and co-parentally exposed, but not paternally exposed embryos, showed presence of PS NPs in the yolk sac at 24 hpf. The transfer of PS NPs between generations in a vertebrate species has implications for both environmental and human health.

After the initial localization to the yolk sac, the PS NPs translocated to the gastrointestinal tract, liver, and pancreas. The gall bladder also showed increased degree of fluorescence in the

maternal and co-parental exposure groups, but this increase was not statistically significant. These results are consistent with those from developing zebrafish that were exposed to PS NPs in the embryo medium (Pitt et al., 2018; Skjolding et al., 2017; van Pomeran et al., 2017). Notably, higher degree of variability in the levels of fluorescence between the different larvae was observed in the current study compared to our previous waterborne exposure study, likely due to unequal distribution of PS NPs in the eggs of the F0 females.

Despite the visible presence of PS NPs in the yolk sac, the survival and incidence of deformities was not significantly different across groups. However, the maternally and co-parentally exposed larvae exhibited bradycardia. This result is congruous with the bradycardia observed previously in our waterborne exposure study (Pitt et al., 2018). The observed bradycardia could be a result of the PS NPs interacting with the cardiac sarcomeres from their localization into cells yielding a change in heart function (Geiser et al., 2005). It should be noted that the pericardium did not have a significantly increased amount of fluorescence in the current study.

Furthermore, co-parentally exposed larvae had fewer inflated swim bladders. The swim bladder of zebrafish typically inflates at 72-96 hpf (Robertson et al., 2007), however the co-parentally exposed larvae had a higher incidence of uninflated swim bladders at 144 hpf. Swim bladder inflation is important as it provides stability for the vertical orientation and migration of the fish, which is critical for predator avoidance, as zebrafish engage in diurnal migration (Robertson et al. 2007; Robertson et al. 2008). Further, the lack of inflation of the swim bladder can also indicate a decrease in feeding efficiency, which is critical as the larva develops and the nutrient-filled yolk sac is resorbed (Goolish and Okutake, 1999; Tait, 1960). Goolish and Okutake (1999) also found that the lack of swim bladder inflation could lead to the development of spinal

curvature in the developing larvae. Overall, this developmental delay as well as the aforementioned bradycardia indicate that co-parental exposure to PS NPs could reduce organismal fitness.

Interestingly, the larval locomotor activity was not affected by parental exposure to PS NPs, even in the co-parentally exposed larvae that displayed an uninflated swim bladder [larvae with uninflated swim bladders are typically hypoactive as they incur a greater metabolic cost for maintaining their position in the water column (Robertson et al., 2007)]. The lack of an effect on locomotor activity contrasts the larval hypoactivity that was reported in previous waterborne exposure studies (Pitt et al., 2018; Chen et al., 2017). However, it should be noted that the relative amount of PS NPs in co-parentally-exposed larvae is smaller relative to the PS NPs amount after a waterborne exposure study. Specifically, in the current study co-parentally-exposed embryos had 7 fold higher fluorescence levels than the control group in the yolk sac at 24 hpf, whereas fluorescence changes of up to 100 fold relative to the control group in the yolk sac at 24 hpf were reported in our previous waterborne exposure study (Pitt et al, 2018).

Oxidative stress markers were also examined in F1 larvae. Similarly to the response in brain and muscle of F0 adults, GR activity was decreased at 96 hpf maternally or co-parentally exposed larvae. In addition, the levels of reduced thiols (both protein and non-protein) were decreased in these same groups. Together, these changes indicate that co-parental exposure to PS NPs could increase offspring's susceptibility to oxidative stress, since there is a decrease in reduced thiol levels and on the other hand a potential inhibition of GR. Currently, it is unknown whether PS NPs can inhibit GR, but such inhibition can have adverse effects, including impaired immune function, lower capacity to detoxify peroxides, oxidative damage, and even lower survival, as reported in bivalves (Franco et al., 2006; Trevisan et al., 2014, Mello et al., 2015). The concentrations of GSH and GSSG, as well as the expression of genes associated with GSH/GSSG

metabolism are tightly regulated during zebrafish early development (Timme-Laragy et al., 2013). It was shown that GR may play a major role during early development, especially after 18 hpf when GSH recycling is increased. Therefore, alterations of GR activity during early development could lead to long-term physiological effects as the larva matures. In addition, reduced GR activity could increase the organism's susceptibility to additional environmental stressors, including contaminants commonly sorbed to plastics such as polychlorinated biphenyls, polycyclic aromatic hydrocarbons, dichloro-diphenyl-trichloroethane and its metabolites, polybrominated diphenyl ethers, alkylphenols, and bisphenol A (Hirai et al., 2011).

Although some effects were noted within the antioxidant system, embryonic mitochondrial bioenergetics were not affected by cross-generational exposure in 24 hpf embryos, similarly to our earlier study (Pitt et al., 2018). As mentioned earlier, oxidative stress could lead to mitochondrial dysfunction. However, it should be noted that mitochondrial bioenergetics analysis was performed in 24 hpf embryos, whereas oxidative stress parameters were assessed at 96 hpf. Therefore, the lack of effects on mitochondrial bioenergetics should be interpreted cautiously, since the possibility of long-term impacts on mitochondrial function cannot be excluded. Further, the potential for toxins known to induce mitochondrial dysfunction, such as PAHs, to sorb to the surface of the nanoplastics in the environment and then accumulate in an organism leading to changes in mitochondrial function should be further investigated (Hartmann et al., 2017; Liu et al., 2016; Meyer et al., 2013). Future studies should examine in more details the potential effects of various plastic types on mitochondrial function throughout development.

In summary, our study indicates that dietary exposure of adult zebrafish to PS NPs could affect the physiology of the offspring. More specifically, our data suggest that PS NPs are maternally transferred to the offspring via accumulation in the eggs of exposed females. This is

probably due to interactions of PS NPs with plasma proteins related to oocytes, facilitating their transport to the gametes. Although PS NPs did not exert overt toxicity to either F0 adults or the F1 embryos/larvae, several changes were noted. In adult female brain as well as both male and female muscle tissues, PS NPs were able to modulate the antioxidant system by decreasing the activity of GR. In embryos/larvae, bradycardia, uninflated swim bladders, and alterations of antioxidant system were noted in maternally- and/or co-parentally-exposed fish. Since PS NPs were also able to modulate the antioxidant system of both F0 adults and F1 larvae by decreasing GR activity and thiol levels, glutathione metabolism could be one potential target of PS NPs. Compromised antioxidant system could increase the susceptibility to other environmental stressors, especially chemicals that can sorb to plastics and be transferred together with nanoplastics within the food chain and/or maternally-transferred to offspring. Compromised antioxidant system could also impair other physiological processes. Although mitochondrial bioenergetics in adults and embryos, as well as larval behavior were not affected in this study, more detailed examination of these and other physiological endpoints is warranted in order to establish the extent of risk that nanoplastics pose to aquatic environment. Taken altogether, these data suggest that PS NPs are capable of affecting developing zebrafish through both a maternal transfer of particles as well as a waterborne exposure.

Declaration of Interests

The authors have no competing interests.

Acknowledgements

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Figures

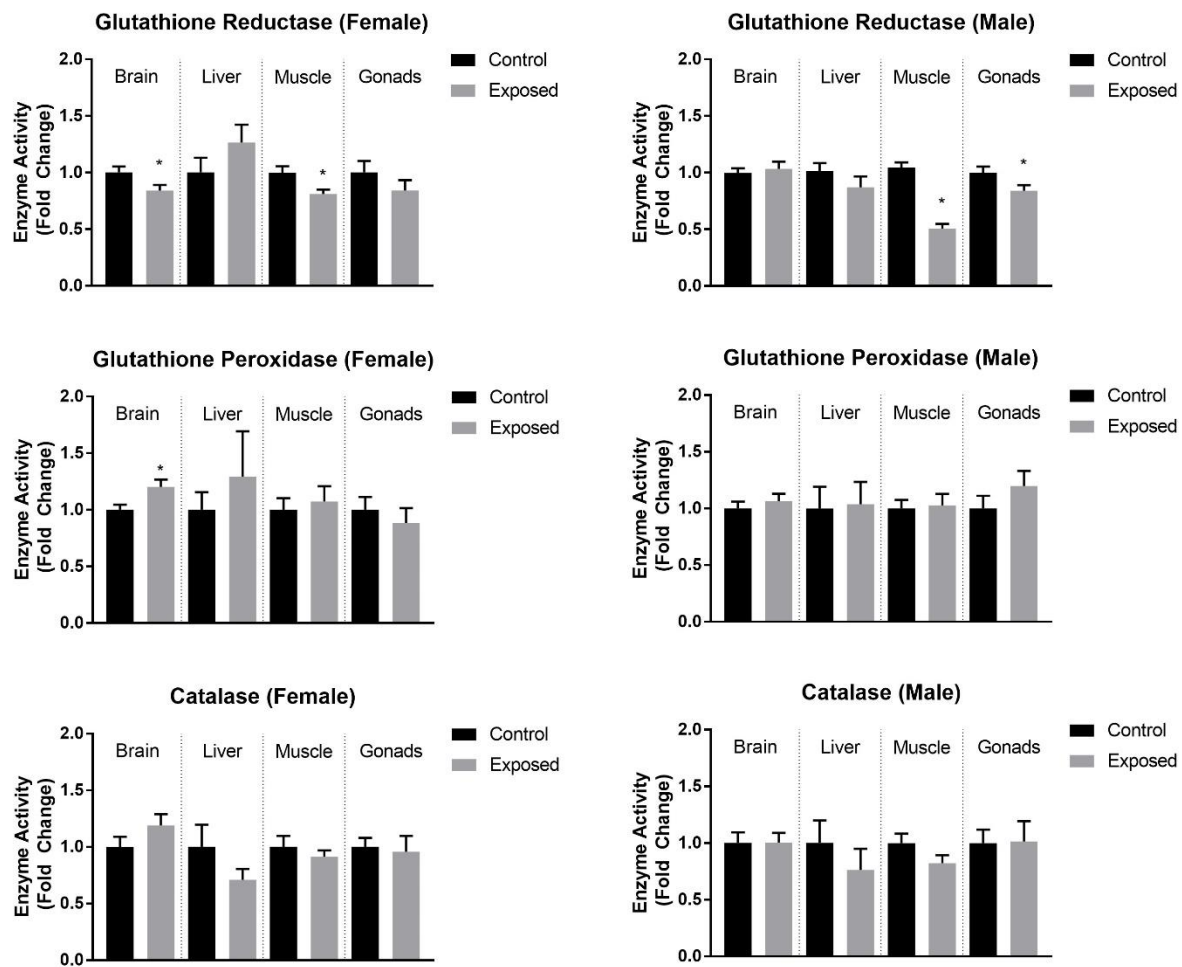


Figure 1. Oxidative stress and antioxidant enzyme activity in tissues from F0 adults dietary exposed to polystyrene nanoparticles.

Activities of the antioxidant enzymes glutathione peroxidase, glutathione reductase, and catalase in tissues of F0 adults. All data are presented as fold change related to the respective control group (mean \pm SEM, n=10-12 across 3 cohorts). Absolute values of the control groups are shown in Supplementary Table 1. Significance was accepted if $p < 0.05$, using one-way ANOVA followed by Tukey's post hoc. An asterisk denotes statistical differences across treatments within each tissue.

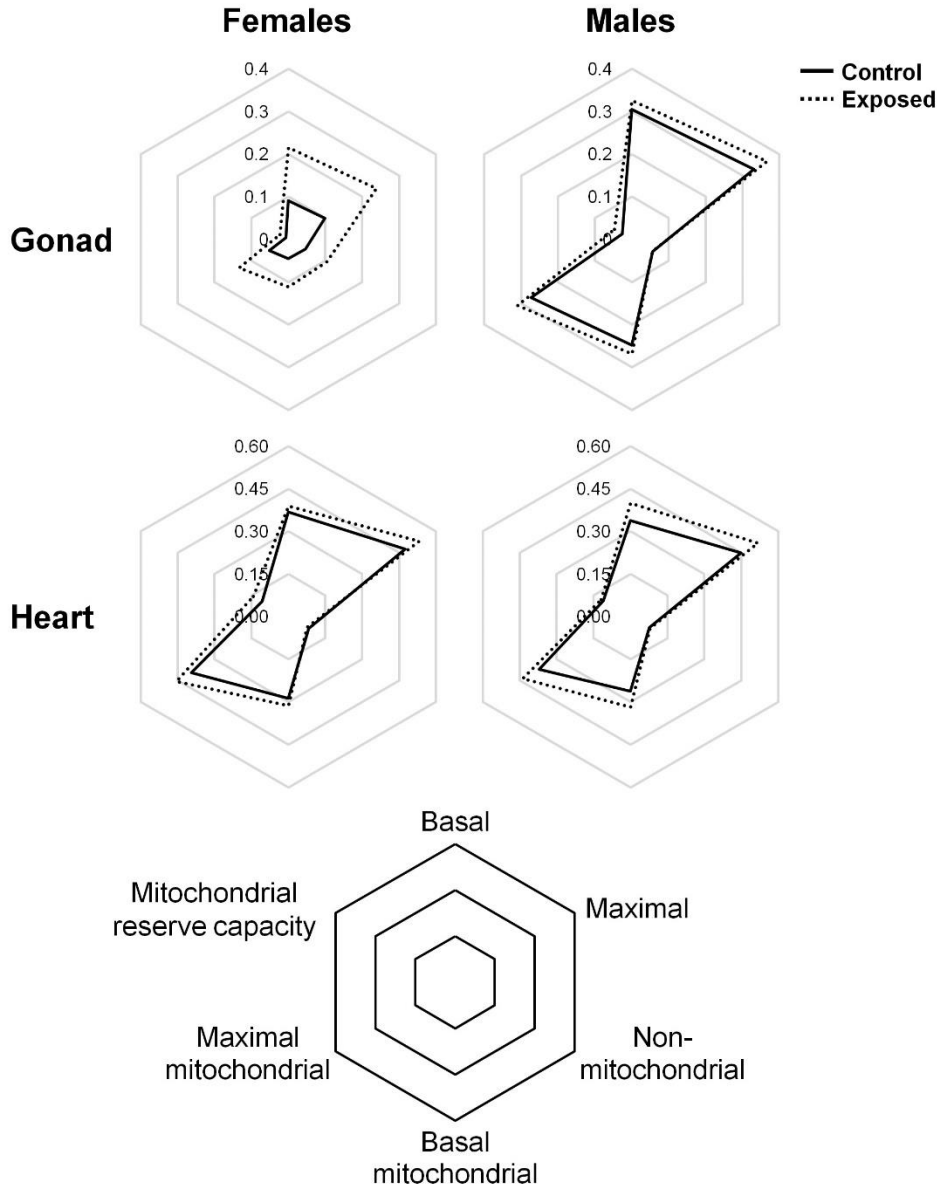


Figure 2. Mitochondrial function and metabolic partitioning in tissues of F0 adult dietary exposed to polystyrene nanoparticles via food diet.

Respiration rates (oxygen consumption rate; OCR) due to various bioenergetics parameters measured *ex vivo* with F0 heart and gonad tissues exposed to PS NPs. Data are shown as $\text{mg O}_2 \text{ h}^{-1} \text{ g}^{-1}$ ($n=4-6$ across 2 cohorts). The parameters measured were: basal respiration, maximal respiration (in presence of FCCP), non-mitochondrial respiration (in presence of rotenone and antimycin), basal mitochondrial respiration (total basal – non-mitochondrial), maximal mitochondrial respiration (total maximal – non-mitochondrial), and mitochondrial reserve capacity (total maximal – total basal). Each point represents a different parameter. Significance was accepted if $p < 0.05$, using one-way ANOVA followed by Tukey's post hoc.

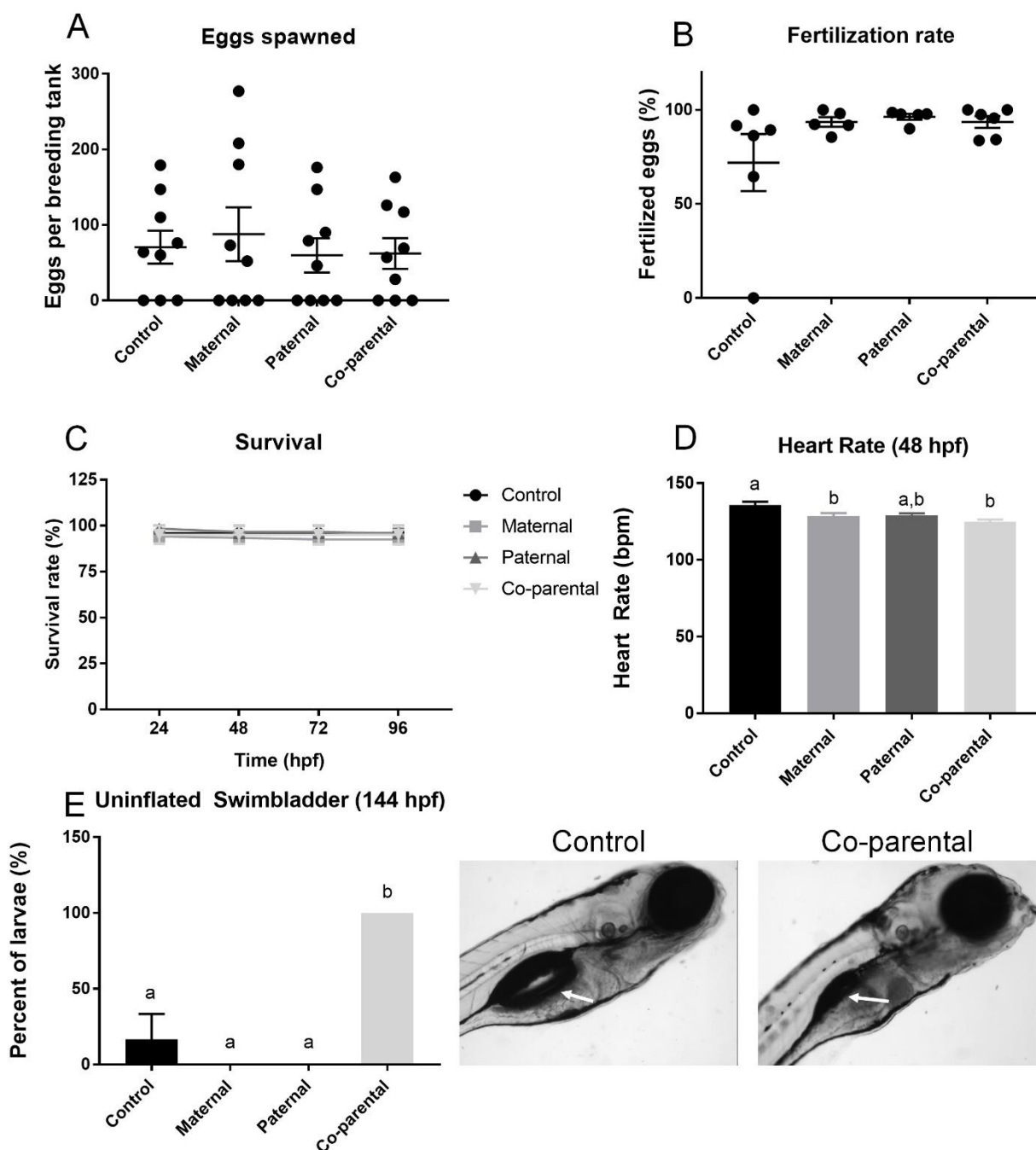


Figure 3. Fecundity, mortality, developmental delays, and cardiotoxicity in F1 zebrafish larvae cross-generationally exposed to polystyrene nanoparticles.

(A) Number of eggs spawned (across 3 cohorts). (B) Fertilization rate of eggs spawned (across 3 cohorts). (C) Survival of larvae exposed to PS NPs from 24-96 hpf (n=4 plates from 2 cohorts). (D) Heart rate at 48 hpf (n=20 across 2 cohorts). (E) Swim bladder inflation was evaluated at 144 hpf (n=6 across 2 cohorts). All data are presented as means \pm SEM. Significance was accepted if $p < 0.05$, using one-way ANOVA followed by Tukey's posthoc. Groups not sharing letters are significantly different.

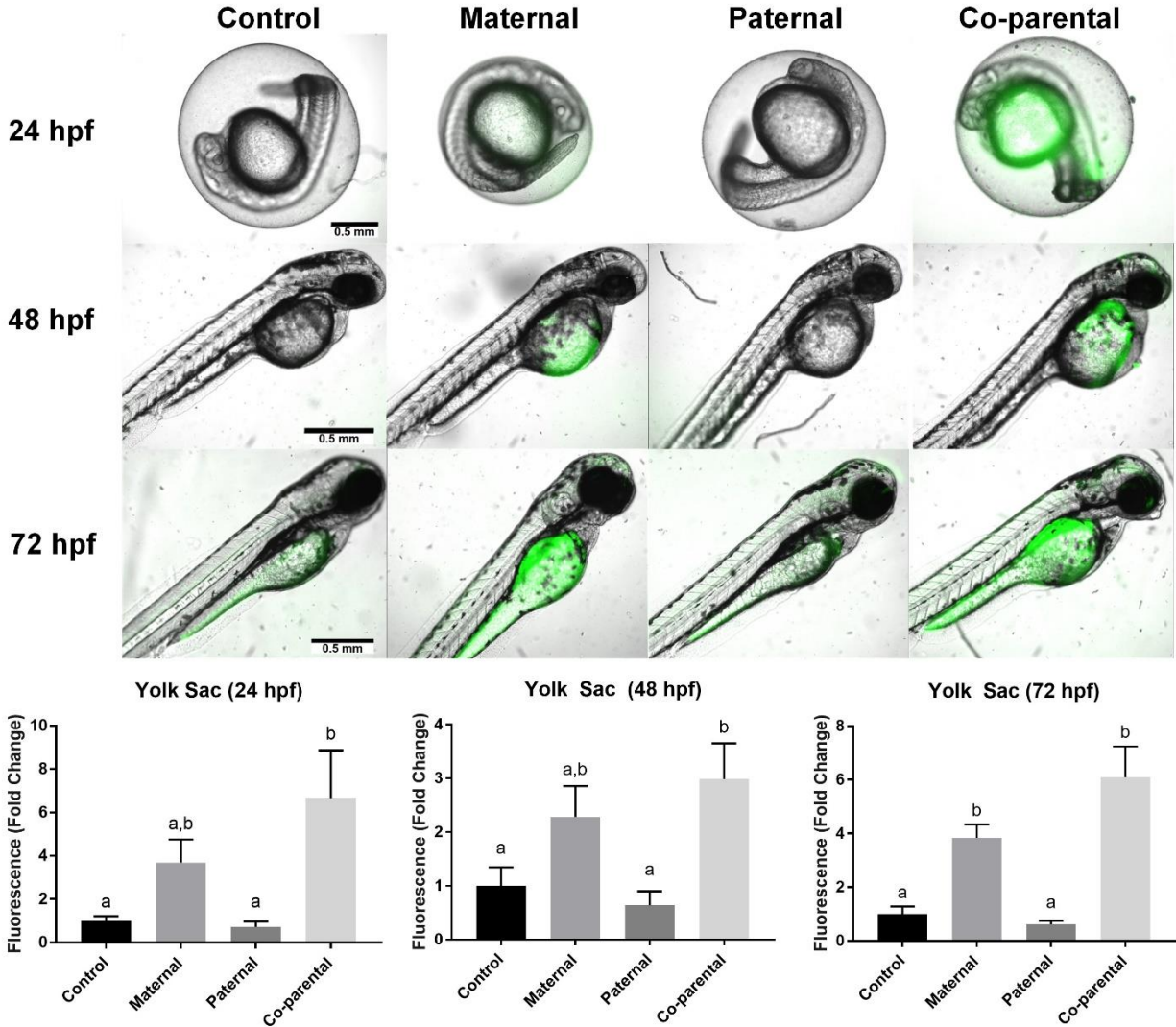


Figure 4. Polystyrene nanoparticles (PS NPs) fluorescence and distribution in 24-72 hpf F1 zebrafish embryos cross-generationally exposed to PS NPs.

On top, representative images (transmitted light merged to green fluorescence) of the zebrafish embryos. At 24 hpf, embryos were imaged with their chorion intact. On the bottom, fluorescence quantification in the yolk sac at each developmental stage, presented as fold change (means \pm SEM, $n=6$ across 2 cohorts). Significance was accepted if $p<0.05$, using one-way ANOVA followed by Tukey's post hoc. Groups not sharing letters are significantly different.

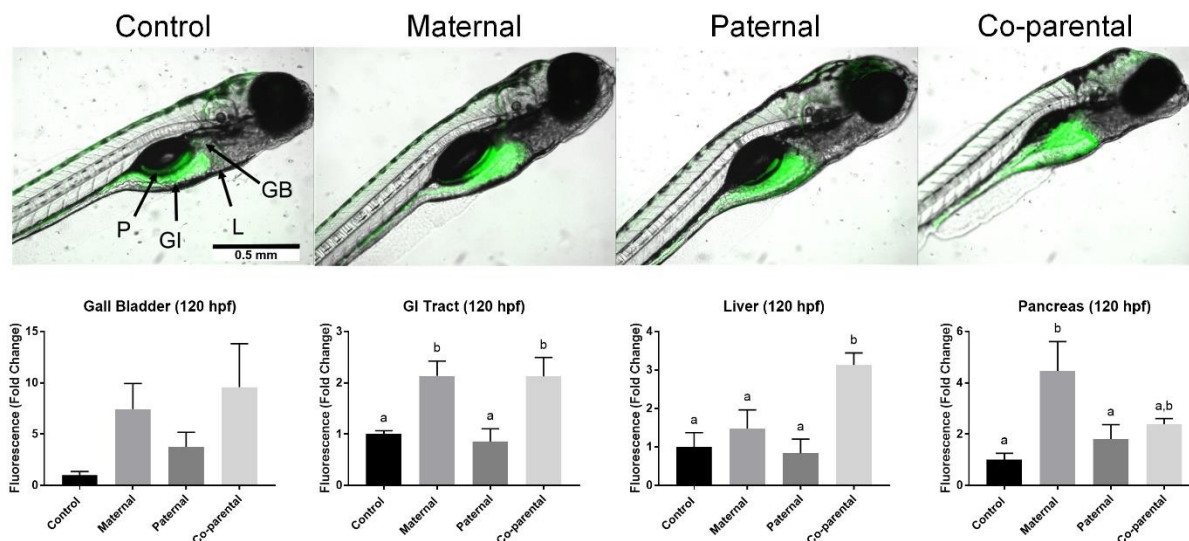


Figure 5. Polystyrene nanoparticles (PS NPs) fluorescence and distribution in 120 hpf F1 zebrafish larvae cross-generationally exposed to PS NPs.

On top, representative images (transmitted light merged to green fluorescence) of the zebrafish larvae. The letters in the control image correspond to various organs analyzed: (GB) gall bladder, (P) pancreas, (GI) gastrointestinal tract, and (L) liver. On the bottom, fluorescence quantification in the gall bladder, pancreas, gastrointestinal tract, and liver at 120 hpf presented as fold change (mean \pm SEM, $n=6$ across 2 cohorts). Significance was accepted if $p<0.05$, using one-way ANOVA followed by Tukey's post hoc. Groups not sharing letters are significantly different.

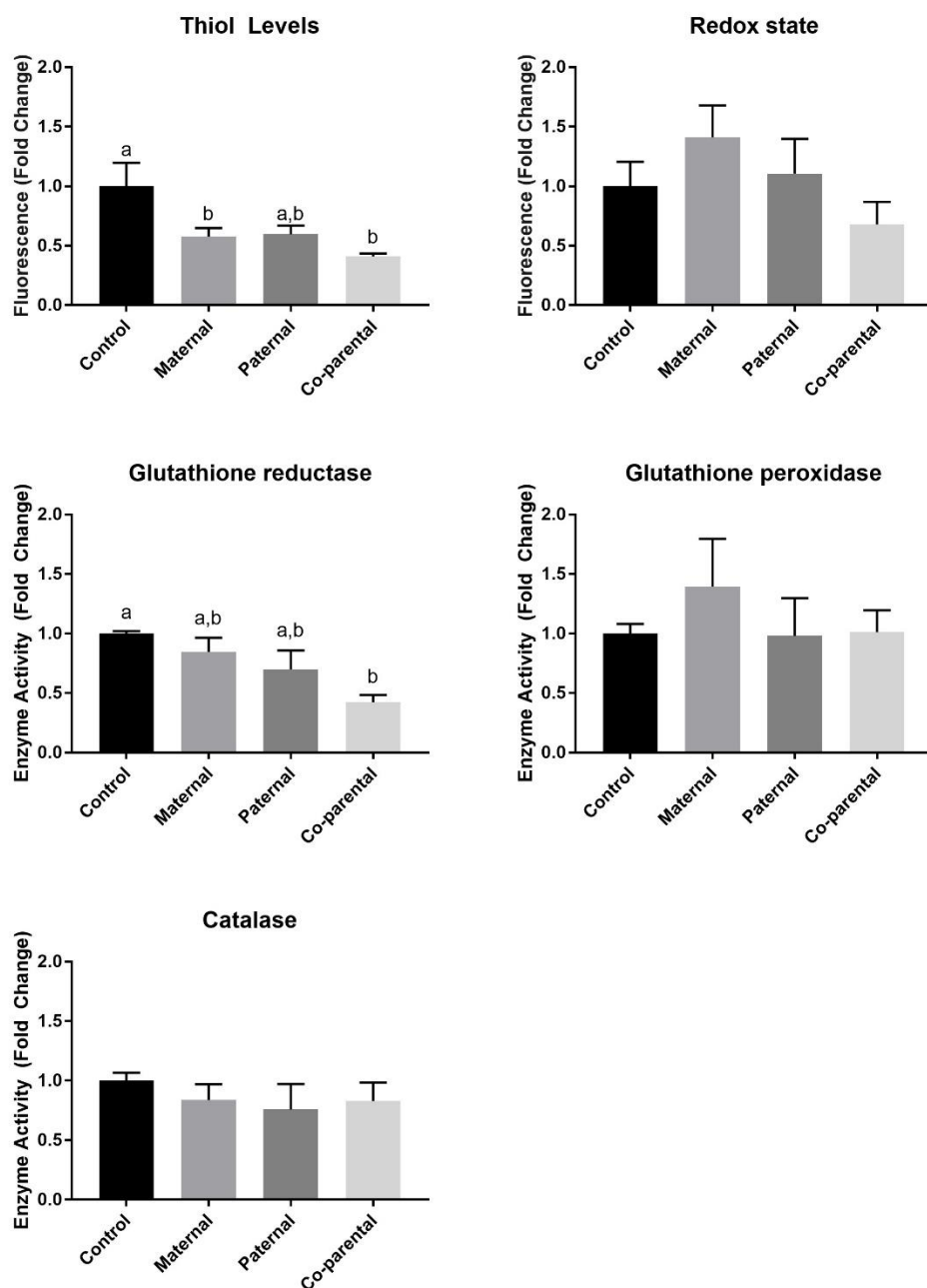


Figure 6. Oxidative stress biomarkers in F1 zebrafish larvae cross-generationally exposed to polystyrene nanoparticles (PS NPs)

Reduced thiol levels and cellular redox state were detected by fluorescence assays and data are presented as fold change (n=16 across 2 cohorts). Activities of the enzymes glutathione reductase, glutathione peroxidase, and catalase at 96 hpf larvae are also presented as fold change (n=5-6 across 3 cohorts). Absolute values of the control groups are shown in Supplementary Table 1. All data are presented as mean \pm SEM. Significance was accepted if $p < 0.05$, using one-way ANOVA followed by Tukey's post hoc. Groups not sharing letters are significantly different.

Supplemental Materials

Maternal transfer of nanoplastics to offspring in zebrafish (*Danio rerio*): a case study with nano polystyrene

Jordan A. Pitt^{1,2}, Rafael Trevisan¹, Andrey Massarsky¹, Jordan S. Kozal¹, Edward D. Levin³, & Richard T. Di Giulio¹

¹Nicholas School of the Environment, Duke University, Durham, NC 27708, USA.

²State University of New York, College of Environmental Science and Forestry, Syracuse, NY, 13210, USA.

³Department of Psychiatry and Behavioral Sciences, Duke University Medical Center, Durham, NC 27710, USA

Table S1. Absolute values of the antioxidant enzyme activities in F0 tissues and F1 larvae.
Values are shown as mean \pm SEM (n=10-12) of the control groups.

Enzyme	Brain		Liver		Muscle		Gonads		Larvae
	Female	Male	Female	Male	Female	Male	Female	Male	
Glutathione Peroxidase ¹	17.2 \pm 1.0	17.6 \pm 1.0	30.1 \pm 5.6	35.2 \pm 6.4	22.4 \pm 2.4	38.4 \pm 2.9	69.0 \pm 15.2	14.6 \pm 2.9	13.9 \pm 2.4
Glutathione Reductase ¹	3.0 \pm 0.2	2.7 \pm 0.1	5.3 \pm 0.8	10.2 \pm 0.9	3.3 \pm 0.3	2.1 \pm 0.1	9.6 \pm 1.2	7.0 \pm 0.9	2.9 \pm 0.1
Catalase ²	3.6 \pm 0.3	3.6 \pm 0.3	36.2 \pm 9.4	56.9 \pm 12.2	5.9 \pm 0.6	6.9 \pm 0.7	28.0 \pm 2.4	14.8 \pm 2.0	4.3 \pm 0.5

¹ Enzyme activity in nmol/min/mg protein

² Enzyme activity in μ mol/min/mg protein

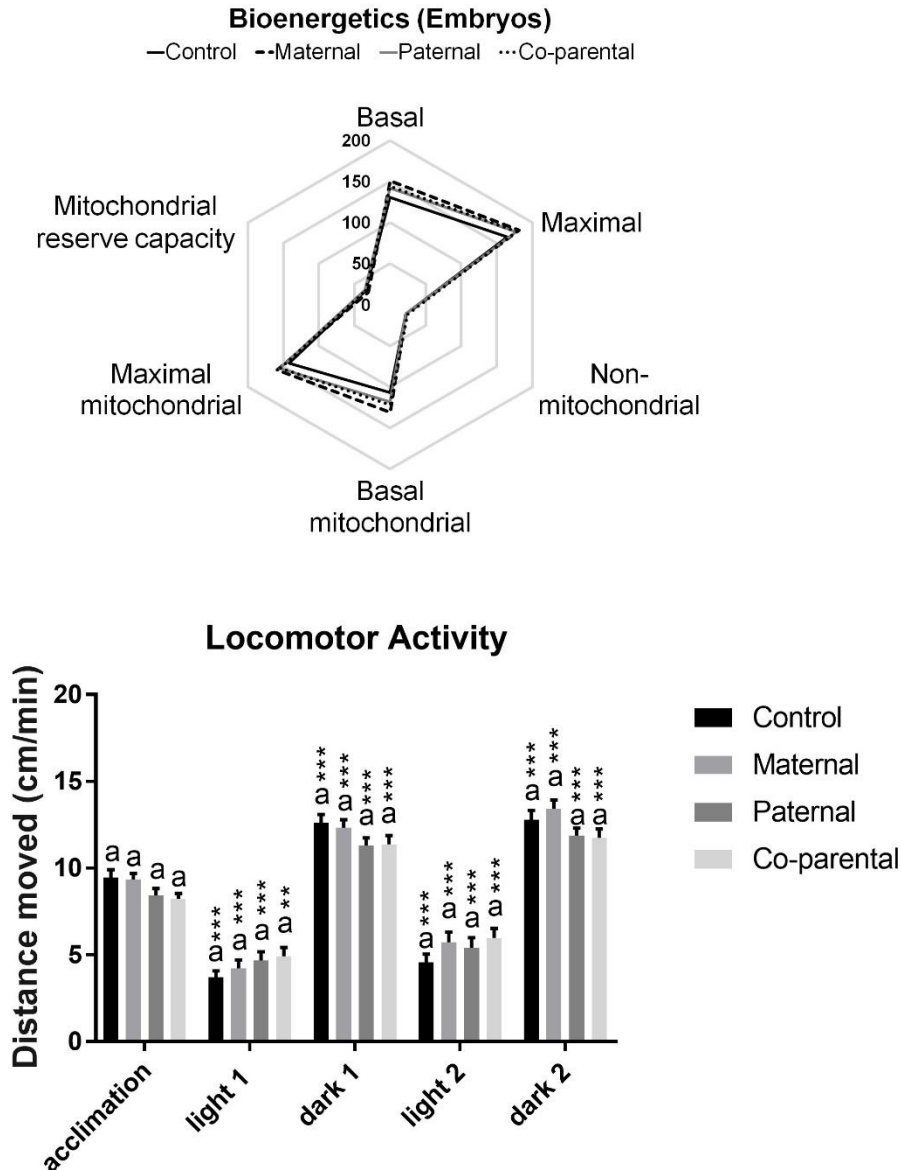


Figure S1. Behavioral toxicity and bioenergetics in polystyrene nanoparticles (PS NPs) cross-generationally exposed zebrafish embryos or larvae

Locomotor activity by light condition of F1 larvae (144 hpf) cross-generationally exposed to PS NPs (n=48 across 2 cohorts). Respiration rates (oxygen consumption rate; OCR) due to various bioenergetics parameters measured *in vivo* with F1 embryos (n=4 for 2 cohorts). The parameters measured were: total basal respiration, total maximal respiration (in presence of FCCP), non-mitochondrial respiration (in presence of rotenone and antimycin), basal mitochondrial respiration (total basal – non-mitochondrial), maximal mitochondrial respiration (total maximal – non-mitochondrial), and mitochondrial reserve capacity (total maximal – total basal). The data are presented as means \pm SEM. Significance was accepted if $p < 0.05$, using one-way ANOVA followed by Tukey's posthoc. Groups sharing letters are considered statistically similar within

each light/dark cycle, while *** ($p < 0.001$) indicates significant differences against the previous cycle for each exposure group.